The HyData Project:

Epidemiology of canine echinococcosis and livestock hydatidosis in the United Kingdom

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Marisol Collins

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This thesis is based on research carried out in the Department of Epidemiology and Population Health, Institute of Infection and Global Health, University of Liverpool. Except for where indicated, this thesis is my own unaided work.

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The zoonotic tapeworm, *Echinococcus granulosus*, mainly cycles between domestic dogs and sheep, but presents a significant risk to human health and is a source of economic loss for livestock industries. In the UK, *E. granulosus* is endemic and historically restricted to mid-Wales, surrounding English border regions and the Hebridean Scottish Islands. *E. equinus*, a non-zoonotic species that mainly cycles between hunting hounds and horses is also endemic. Following a fall in *E. granulosus* prevalence after a hydatid control programme in mid-Wales in 1979-89, recent surveys report a re-emergence of infection in Wales and case reports suggest a wider distribution beyond known prevalence hotspots. Information on the molecular epidemiology of *Echinococcus* spp., in particular *E. granulosus*, to support this emergent and re-emergent picture is needed. This thesis uses questionnaires, coproantigen ELISA, PCR, DNA sequencing and histopathology to study *Echinococcus* spp. in UK farm dogs, hunting hounds, zoo canids and livestock, collectively called the HyData project. The thesis finds evidence that *E. granulosus* distribution extends far beyond known hotspots in the UK and shows that *E. granulosus* is involved in the re-emergence of infection in Wales. In a study of 46 UK sheep farms, 17.4% housed dogs positive for *Echinococcus* spp. coproantigen, with a widespread national distribution; 10.9% had dogs positive for *E. granulosus* coproDNA in Wales, reporting for the first time in the North of England, Scotland and Northern Ireland. Positive results were significantly associated with location in Wales ($p<0.05$), supporting a picture of re-emergent *Echinococcus* spp. transmission in that region. In a study of 32 UK hunting hound packs, 9.4% hunts tested positive for *Echinococcus* spp. coproantigen, reported for the first time in the North West and South West of England and the Scottish Borders. A further pack in the North West of England is the first reported positive for *E. granulosus* coproDNA. In a study of canids and hyaenids in 22 UK zoos, 22.7% of collections (all in England) housed species testing positive for *Echinococcus* spp. coproantigen, with the first reported UK cases in African hunting dog, European grey wolf, Iberian wolf, Arctic fox, Black-backed jackal collections and the first reported UK case of *E. equinus* by coproPCR in the European Wolf. A survey of 87 hydatid cases (and 261 controls) in cattle and sheep slaughtered at 15 abattoirs in England and Wales reported 7.1% of samples positive for *Echinococcus* spp. on PCR; Of samples submitted as hydatid, 32.9% were confirmed as *Echinococcus* spp. by PCR and 26.9% as *E. granulosus* by DNA sequencing. Matching of cattle movement records from 23 PCR-confirmed *E. granulosus* cases reported cattle travelling in Wales or adjacent counties as significantly more likely to have hydatid disease (OR=15.47, $p<0.0001$). Four cases had never entered Wales: two adjacent in Gloucestershire and Herefordshire and two further afield in Staffordshire and North Yorkshire/Humber. A proof-of-concept exercise to evaluate meat inspection for hydatidosis estimated a diagnostic sensitivity of 30.68%, (95%CI: 11.91-49.44) and specificity of 99.48%, (95%CI: 99.48-100.0) suggesting likely underreporting of disease at meat inspection, although more data are needed to optimise this calculation. Factors associated with increased risk of *Echinococcus* spp. infection were common to all canine study groups. Over a third of participating farms (36.2%), 79.1% of hunts and 80% of zoos reported feeding raw meat and offal from fallen stock to canines; 44.7% of farms reported witnessing farm dogs scavenge carcases of fallen stock on-farm. Only 57.5% of farms, 29.6% of hunts and 23.5% of zoos were administering a suitable wormer at a minimum dose for *E. granulosus* control. In farm dogs, sub-optimal worming was significantly associated with a positive *E. granulosus* PCR result ($p<0.05$). Routine faeces collection was reported by all hunts, all zoos and 55% of farms; however, 44.4% of zoos, 50% of hunts and 83% of farms then reported disposing of faeces by means, such as muck heaps and farm fields, which could potentially contaminate agricultural land with *Echinococcus* spp. eggs if used as unprocessed fertilizer, posing an under-researched risk to public and animal health. The thesis findings call for effective and targeted *E. granulosus* control in dogs and livestock and for nationwide prevalence studies to further explore a renewed public health risk. As an exercise in baseline data gathering, the approach of the HyData project and the thesis findings would inform the planning phase of a UK hydatid control programme.

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I was under no illusion that researching *Echinococcus* where it exists at low levels using laborious and problematic diagnostic tests would be a challenge. I likened it to looking for a needle in a haystack while wearing dark glasses. It is a parasite that can be good at not being found, both in the field and in the laboratory, but we found it, and that is a meaningful addition to what we know.

During this research adventure, I have been fortunate to have a supportive supervisory team; Dr Eleni Michalopoulou, Dr Phil Jones, Dr John Mcgarry and Prof. Mike Rogan. Their expertise in epidemiology, public health, parasitology, cestode diagnostics and PhD supervision was invaluable in such a varied project. I thank them all for their kindness and patience towards a student who I'm sure was exasperating at times. I thank Prof. Debbie Archer and Dr Dan Neill as my Progress Assessment Panel for their mentorship. I feel privileged to have moved forward in my research career with the help of all these excellent teachers.

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The thesis has been an exercise in multidisciplinary collaboration. I thank the FSA, the NSA, BIAZA and the Hunting Office for their time and willingness to take part in this research, and to the farmers, meat hygiene inspectors, official vets, hunt staff

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I thank Dr Lesley Bell-Sakyi, my current boss, for her patience and support as I juggled writing and working at my new research post. I hope to build on the work of this thesis and the research it describes, as there is much to do to better understand *Echinococcus* and address it in parts of the world where it impacts greatly on animal and public health.

My friends and colleagues at Liverpool University on both sides of the Mersey have given me support, counsel and clarity along the way. They knew when it was time to sit down, talk the day through, laugh and worry less. Many had completed their doctorates and pointed to the potholes and eddies to navigate along the way and others shared the journey with me. These talented friends have been an inspiration and I am immensely proud of all they have achieved in their research fields. I could not have asked for better friends, even if I am old enough to be a parent to some (although they never made me feel old). I want to especially thank Charlotte, Emma, Alex, Lucy, Gabby, David, Rita, Pete, Arturo, Maria, Raquel, Camilla, Cat McCarthy, Cat Wilson, Tasha and Lisa.

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“Echinococcus..? It’s a bloody hard parasite to work with”

Dr Tony Bodell,
Specialist Technician (Molecular),
Cestode Zoonosis Research Group and Cestode Diagnostics Ltd.,
University of Salford. 25.6.19.

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ABBREVIATIONS

ABP	Animal By-Products
AE	alveolar echinococcosis
AHDB	Agriculture and Horticulture Development Board
APHA	Animal and Plant Health Agency
APHIS	Animal and Public Health Information System
AVM-GSL	Approved veterinary medicine - general sale
AZA	Association of Zoos and Aquariums
BRP	Better Returns Programme
BIAZA	British and Irish Association of Zoos and Aquariums
bTB	Bovine tuberculosis
CCIR	Collection and Communication of Inspection Results
CE	Cystic echinococcosis
CHA	The Council of Hunting Associations
CL	Containment Level (ABP)
coproELISA	Coproantigen Enzyme-Linked Immunosorbent Assay
coproPCR	Coprological Polymerase Chain Reaction
COSHH	Control of Substances Hazardous to Health
CTS	Cattle Tracing System
DALY	Disability-Adjusted Life Year
EFSA	European Food Safety Authority
ESCCAP	European Scientific Counsel Companion Animal Parasites
FAQ	Frequently Asked Questions
FCI	Food Chain Information
FMD	Foot and Mouth Disease
FSA	Food Standards Agency
FSS	Food Standards Scotland
GI	Gastrointestinal
H&E	Haematoxylin and eosin
HSE	Health and Safety Executive
IGH	Institute of Infection and Global Health (Liverpool University)
IHA	Indirect hemagglutination test
IQR	Inter-quartile range
ISDS	International Sheep Dog Society
ITL	Inspection Team Leader
MAFF	Ministry of Agriculture Food and Fisheries
MFHA	The Masters of Foxhounds Association
MHIs	Meat Hygiene Inspectors

NADIS	National Animal Disease Information Service
NADH	NADH dehydrogenase subunit
ND1	NADH dehydrogenase subunit
NFA-VPS	a vet, pharmacist or trained specialist without prescription
NFSCo	National Fallen Stock Company
NFU	National Farmers Union
NFUS	National Farmers Union Scotland
NOAH	National Office of Animal Health
NPV	Negative predictive value
NSA	National Sheep Association
OD	Optical density
OIE	World Organisation for Animal Health
OVs	Official Veterinarians
PCR	Polymerase Chain Reaction
PCR-RFLP	Restriction Fragment Length Polymorphism PCR
PE	Polycystic echinococcosis
PFMA	Pet Food Manufacturers' Association
PHE	Public Health England
PHW	Public Health Wales
POM-V	Prescription only medicine - Veterinary
PPV	Positive predictive value
RADAR	Rapid Analysis and Detection of Animal-related Risk
ROC	Receiver Operator Characteristic
RTA	Red Tractor Assurance
SAVSNET	Small Animal Veterinary Surveillance Network
Se	Sensitivity
SEM	Standard error of the mean
Sp	Specificity
SQP	Specially qualified person
SRM	Specified Risk Material
SVS	Sheep Veterinary Society
TP	True prevalence
TSEs	transmissible spongiform encephalopathy
VAWM	Veterinary Association for Wildlife Management
WHO	World Health Organization
ZIMS	Zoological Information Management System

Chapter 1

Introduction

1 Introduction

1.1 Overview

Echinococcosis is a neglected zoonotic disease caused by cestode parasites of the genus *Echinococcus* (Eckert *et al.*, 2002; Kern *et al.*, 2017). There are three broad clinical manifestations of echinococcosis: cystic echinococcosis (CE), caused by *E. granulosus*, *E. ortleppi* and *E. canadensis*, alveolar echinococcosis (AE) caused by *E. multilocularis* and polycystic echinococcosis (PE), caused by *E. vogeli* and *E. oligarthus* (Eckert and Deplazes, 2004a; Moro and Schantz, 2009b; Agudelo-Higuita, Brunetti and McCloskey, 2016; Kern *et al.*, 2017).

Human CE has a wide global distribution and accounts for over 95% of the estimated 2-3 million cases of echinococcosis worldwide (Budke, Deplazes, & Torgerson, 2006; Craig, Rogan, & Allan, 1996). The non-monetary global burden of disease associated with CE has been estimated at 285,400 lost daily-adjusted life years (DALYs) annually, rising to 1,010,000 DALYs when accounting for underreporting (Budke *et al.*, 2006), a figure greater than the comparative for Dengue or Chagas disease (Budke *et al.*, 2006; Craig *et al.*, 2007). Global economic impact due to diagnosis and treatment costs of human disease has been estimated at $763 million US dollars and losses to the livestock industry estimated at $2,190 million US dollars annually (Budke *et al.*, 2006). Although AE is less common than CE, with distribution mainly in the northern hemisphere and a global disease burden of approximately 18,200 case per year, a higher morbidity and mortality associated with disease results in an estimated 666,000 lost DALYs per year (Torgerson *et al.*, 2010). PE, also termed neotropical echinococcosis, is confined to Central and South America, where fewer than 150 cases have been described (Eckert & Deplazes, 2004; Moro & Schantz, 2009b).

The public health risk of CE has been eliminated or significantly reduced in many previously endemic regions through integrated control programmes targeting deworming of dogs, meat inspection, health education and effective surveillance (Palmer *et al.*, 1996a; Lloyd, Walters and Craig, 1998; Craig *et al.*, 2007; Lembo *et al.*, 2013; Craig *et al.*, 2017). Despite global control efforts, CE remains a serious

neglected zoonosis, particularly in resource-poor parts of the world where prevention and control are difficult to implement and sustain. Difficulties in control and elimination also remain in resource-rich nations, where shortcomings in control interventions have led to a re-emergence of disease (Craig *et al.*, 2007; Lembo *et al.*, 2013; Craig *et al.*, 2017).

1.2 Taxonomy of *Echinococcus spp*.

The taxonomy of *Echinococcus* spp. is contentious and remains the subject of ongoing and conflicting revisions in the literature (Moro & Schantz, 2006; Romig, Ebi, & Wassermann, 2015; Thompson, 2008; Thompson & McManus, 2002). Despite the ongoing review of taxonomy, the general current view, based on biology, epidemiology and molecular genotyping, recommends the classification of nine species (Craig *et al.*, 2015; Lymbery, 2017; Otero-Abad & Torgerson, 2013). Within these nine species are several strains re-classified to species status from a group of previously recognized sub-specific genotypes of *E. granulosus* (G1-G10). The current nine recommended species are *E. granulosus sensu strictu* (s.s) (previously G1-G3), *E. equinus (previously G4)*, *E. ortleppi (previously G5)*, *E. canadensis* (previously G6-G10), *E. multilocularis*, *E. vogeli*, *E. oligarthus*, *E. shiquicus* and *E. felidis* (Alvarez *et al.*, 2014; Craig *et al.*, 2015; Marion Hüttner *et al.*, 2008; Otero-Abad & Torgerson, 2013; Torgerson, 2013).

The G1 genotype (sheep strain) is the most common strain of *E. granulosus* and is responsible for the majority of zoonotic CE (Craig *et al.*, 2015). *E. granulosus* G1 and *E. equinus* are known to be endemic to the UK (Boufana *et al.*, 2015; Deplazes *et al.*, 2017). Neither parasite is notifiable. *E. equinus* is generally not thought to be zoonotic (McManus, Thompson, & Lymbery, 1989; Moro & Schantz, 2006; Romig, Dinkel, & Mackenstedt, 2006) although a recent putative case has been reported in Asia (“Discontools: Echinococcosis,” 2019). *E. multilocularis*, although an important zoonotic parasite, is not known to be present in the UK and is classed as a notifiable disease (The Zoonoses (Monitoring) (England) Regulations 2007).

1.3 Cystic echinococcosis, *E. granulosus* and *E. equinus*

The ecology and epidemiology of *E. granulosus* and *E. equinus* in the UK are described in detail and in context across each of the four individual study chapters. Therefore, they are not addressed at length here. This introduction provides an overview of the biology of the parasite, the burden of disease and risk factors for human and animal infection; it explains the rationale for this research, the hypotheses the research explores and the aims of the overarching project.

Globally, CE is maintained primarily through a two-host lifecycle of domestic dogs and domestic ungulates, typically livestock species (Eckert and Deplazes, 2004a; Romig *et al.*, 2017). It remains a persistent zoonotic problem in rural areas where livestock and humans cohabit with dogs that are fed or can scavenge raw meat products, particularly offal (Torgerson and Budke, 2003; Otero-Abad and Torgerson, 2013; Romig *et al.*, 2017).

Human CE is characterised by the formation of hydatid cysts, mainly in the liver and lungs, which can cause serious morbidity and mortality if left untreated (Moro & Schantz, 2009a). Hydatid cysts in livestock are associated with poor growth, reduced meat and milk production and rejection of organs at meat inspection (Eckert and Deplazes, 2004a; Budke, Deplazes and Torgerson, 2006; AHDB, 2018).

Domestic dogs are a definitive host for several *Echinococcus* species of public health importance. Asymptomatic infected dogs harbour adult worms in the intestine and can infect humans via eggs shed in faeces. Livestock mammals, particularly sheep, and small rodents represent the most important intermediate hosts for *E.granulosus* and *E. multilocularis* respectively (Torgerson *et al.*, 2003), the two most important zoonotic species within the genus.

1.3.1 Lifecycle of *E. granulosus* and *E. equinus*

E. granulosus G1 maintains a life cycle that includes canids, most commonly domestic dogs (Deplazes *et al.*, 2011; Otero-Abad & Torgerson, 2013) and wolves (Guerra *et al.*, 2013; Sobrino *et al*., 2006) as the primary host and sheep as the main secondary

host, although many other species, including cattle, pigs, goats and humans can also be infected (reviewed by Eckert and Deplazes, 2004a). Within such hosts, the larval stages of *E. granulosus* can form large hydatid cysts in the liver, lungs and other sites leading to tissue damage and disease. Dogs typically become infected by scavenging or being fed raw infected livestock meat and offal and will carry the parasite in the intestine without any signs of disease. Tapeworm eggs passed in faeces of dogs are orally ingested by the intermediate host during grazing, thus completing the lifecycle of the parasite. Humans can become infected through accidental ingestion of eggs in contaminated soil, water or food, or by direct contact with an animal host. The pre-patent period of *E. granulosus* is between 42 and 45 days (Craig *et al.*, 2017a). *E. equinus* mainly maintains a transmission cycle between domestic dogs and horses, although transmission also exists between wild canids and equids (Thompson, 2008; Romig *et al.*, 2017). The pre-patent period for *E. equinus* in the definitive canid host is approximately 70 days (Cook, 1989). The lifecycles of *E. granulosus* and *E. equinus* are described in greater detail in Figure 1-1.

1.3.2 CE in humans

Human CE manifests as the development and growth of fluid filled cysts in affected organs, mainly the liver and lungs, though the abdominal cavity, heart, nervous system and other locations can be affected (Moro & Schantz, 2009b). Due to the slow growth of cysts, disease may not manifest until many years post-infection, when the size and number of lesions cause organ dysfunction where they reside (Kern *et al*., 2017)

Detection of cysts using imaging methods remains the mainstay in diagnosis of human disease e.g. radiography, ultrasonography, computed tomography and magnetic resonance imaging (Craig *et al.*, 2007; Moro and Schantz, 2009b; Kern *et al.*, 2017) An international classification of cysts by ultrasound has been developed

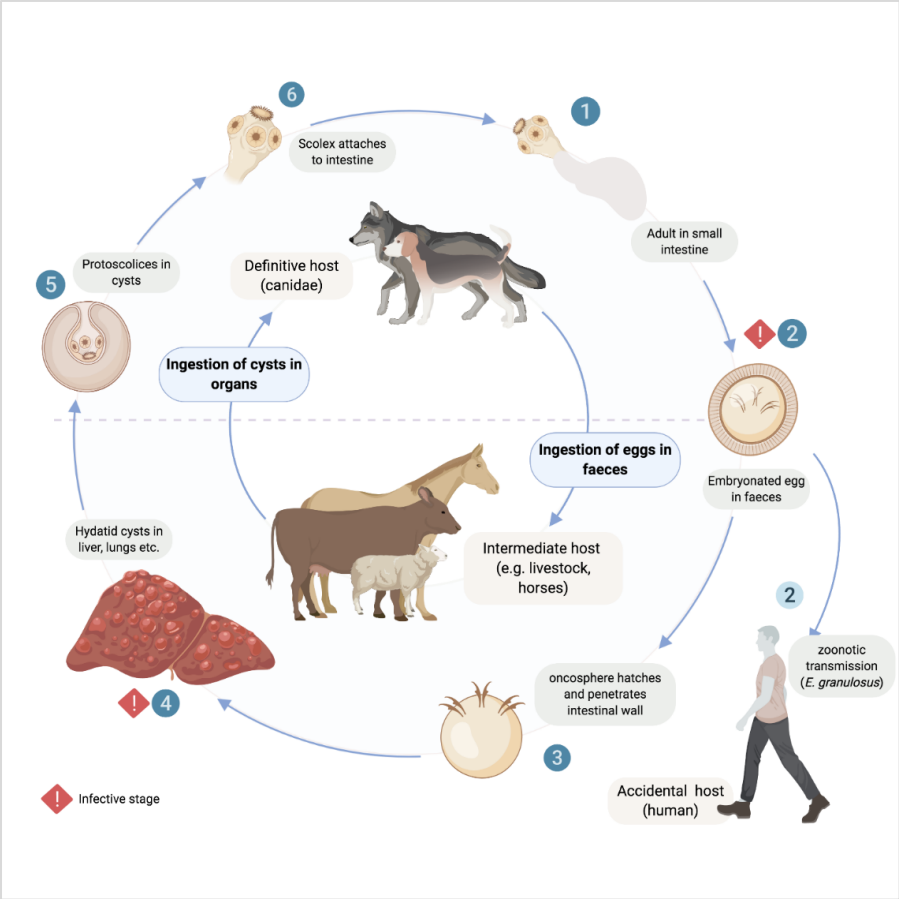

Figure 1-1. Life cycle of E. granulosus and E. equinus endemic to the UK. ❶Adult tapeworms reside in the small intestine of canid hosts. After 42-45 days post-infection in E. granulosus and approximately 70 days in E. equinus, eggs are released from gravid proglottids are passed in the faeces ❷. Eggs are immediately infectious and after ingestion by the intermediate host (sheep, cattle, horses and others), typically during grazing contaminated pasture, the egg hatches in the small bowel and release an oncosphere ❸, which penetrates the intestinal wall. Oncospheres then migrate via the circulatory system to their predilection sites, typically the liver and lungs, although other organs can be affected. Here, the oncosphere develops into a cyst ❹, forming the infective metacestode stage of the parasite, which enlarges gradually, producing protoscoleces within brood capsules and further daughter cysts within the cyst interior ❺. The definitive canid host is infected by eating raw organs containing fertile cysts, usually through scavenging or being fed raw carcass material. After ingestion, the protoscoleces evaginate ❻ and attach to the intestinal mucosa, where they develop into the adult worm. Mature gravid worms will shed their terminal proglottid containing eggs into the intestinal lumen, to be passed in faeces, completing the lifecycle. Humans can be infected by E. granulosus (though not E. equinus) by ingesting eggs ❷ via contact with the faeces, fur or saliva of infected canids or via eggs contaminating fruit, vegetables or water sources. Image created by the author with Biorender.com after Moro and Schantz (2009a).

by the WHO expert group in echinococcosis (WHO, 2016). Laboratory methods to detect specific serum antibodies e.g. enzyme-linked immunosorbent assay (ELISA)

and indirect hemagglutination test (IHA) provide a sensitive confirmatory step, but can have low specificity due to cross-reaction with AE and other taeniid diseases (Craig *et al.*, 2007; Kern *et al.*, 2017). Histopathology and molecular diagnosis by detection of *Echinococcus* spp. antigen or DNA from cysts can be used to identify the parasite to species level (Siles-Lucas *et al.*, 2017).

A structured and staged approach to treatment is guided by the WHO cyst classification system and can involve one or a combination of surgery, percutaneous drainage, medical treatment and a 'watch and wait' approach if infection appears in remission (Moro and Schantz, 2009b; Barnes *et al.*, 2012; Mandal and Mandal, 2012; WHO, 2016; Kern *et al.*, 2017). However, CE remains challenging to treat, with difficult access to lesions, variable responses to treatment, risk of severe adverse reactions and unpredictable prognoses requiring long-term follow-up care (Craig *et al.*, 2007; Kern *et al.*, 2017).

Humans become infected through the accidental ingestion of parasite eggs through petting dogs, allowing dogs to lick the face, contact with dog faeces, contaminated soil or through consumption of food or water contaminated tapeworm eggs (Jenkins, Schurer and Gesy, 2011; Torgerson, 2013; Romig *et al.*, 2017; Vuitton, Zhang and Giraudoux, 2017). Risk factors for human CE include a rural occupation, history of dog ownership, poor education, age and water source access (Mastin *et al.*, 2011; McManus, Zhang, Li, & Bartley, 2003; Thompson & Jenkins, 2014). The finding of a cyst-like mass in a person with a history of exposure to farm dogs in an endemic area supports the diagnosis of CE (Moro & Schantz, 2009a). Human behaviour, such as a close animal-human bond and the purposeful feeding of dogs with raw offal help to perpetuate the domestic cycle of *E. granulosus* (Craig *et al.*, 2007; Craig, Rogan, & Campos-Ponce, 2003; Deplazes *et al.*, 2011). Through a close association with dogs and suboptimal hand hygiene, it has been suggested that children are at particular risk of CE infection (King & Hutchinson, 2007).

1.3.3 CE in animals

1.3.3.1 Echinococcosis in the definitive canine host

Domestic and wild canine hosts maintain *Echinococcus* spp. infection in the intestines without clinical signs of disease and will shed eggs in faeces that are immediately infective to the intermediate host (Craig *et al.*, 2017; Thompson, 2017). Dogs appear to be highly susceptible to infection with all *E. granulosus* genotypes (Cardona & Carmena, 2013) with domestic dogs being the key host in the transmission of CE worldwide (Craig *et al.*, 2015).

Many risk factors for infection in definitive canine hosts have been identified, and these have been recently and extensively reviewed (Otero-Abad and Torgerson, 2013; Romig *et al.*, 2017). One of the most important and commonly identified risk factors for canine infection is access to infected raw animal products, particularly offal, either through purposeful feeding of raw meat products (Carmona *et al.*, 1998; Moro *et al.*, 1999; Buishi *et al.*, 2005b; Acosta-Jamett *et al.*, 2014) or increased access to the carcasses of fallen stock and discarded home slaughter waste (Acosta-Jamett *et al.*, 2010, 2014) and the ability to roam free to find them (Buishi *et al.*, 2005b; Mastin *et al.*, 2011). Other reported determinants include dogs living in rural locations with likelihood of contact with livestock (Acosta-Jamett et al., 2010; Parada et al., 1995) and dog type (farm/working/stray/hunting) (Moro *et al.*, 1999; Shaikenov *et al.*, 2003; Buishi *et al.*, 2005a; Lett *et al.*, 2018). Several studies report a higher risk of *E. granulosus* in younger dogs (Buishi *et al.*, 2005b; Acosta-Jamett *et al.*, 2010; Inangolet *et al.*, 2010), which may suggest an effect of acquired immunity, or an age-related behaviour (Buishi *et al.*, 2005b; Torgerson, 2008).

Surveillance of echinococcosis in dogs is considered the most effective method to evaluate human risk and the effectiveness of control efforts (Craig & Larrieu, 2006; Eckert, Gemmell, Meslin, & Pawłowski, 2001). Post mortem examination of the small intestine for the presence of adult tapeworms using sedimentation and counting techniques is the gold standard method of *Echinococcus* spp. detection in dogs (Craig et al., 1996; Eckert & Deplazes, 2004). Reported sensitivity and specificity of necropsy are high (>97%), although specificity can be affected by coinfection with *E.*

multilocularis due to similar morphology (Craig *et al.*, 2015). For ethical reasons, necropsy is often not suitable for surveillance in populations of dogs (Hartnack *et al.*, 2013). Purgation of gut contents using orally-dosed arecoline plant extracts is a gold standard *ante-mortem* test for detection of adult worms (Craig *et al.*, 1996; Eckert *et al.*, 2001). Although highly specific (Craig *et al.*, 1995), it is poorly sensitive and has been shown to diagnose less than 50% of infected cases after a single treatment (Allan *et al.*, 1991). The method requires trained personnel, is logistically difficult and time consuming to undertake, and has welfare implications for dogs due to distress and risk of complications (Ziadinov *et al.*, 2008). Serological techniques to diagnose definitive host infection have shown limited success in studies to date (Eckert and Deplazes, 2004a; Craig *et al.*, 2017). There is poor correlation between antibody titre and worm burden and cross reaction with other cestode parasite infections (Deplazes *et al.*, 1992). Antibody levels in naturally infected canids can vary and do not differentiate between active or recent infection (Allan *et al.*, 1991). Faecal egg microscopy is rapid and simple, though will not differentiate between *Echinococcus* spp. and other taeniid cestodes and lacks sensitivity as eggs are not continuously shed (Allan *et al.*, 1991).

ELISA methods to detect genus-specific *Echinococcus* spp. coproantigens (coproELISA) using polyclonal or monoclonal antibodies to detect excretory-secretory products from *Echinococcus* proglottids (e.g. Deplazes *et al.*, 1992; Buishi *et al.*, 2005b; Allan and Craig, 2006; Dalimi *et al.*, 2010) or protoscoleces (e.g. Benito and Carmena, 2005) have been developed. CoproELISA allows high sample throughput, is relatively inexpensive and reports reasonable sensitivity (78-100%) (Allan & Craig, 2006; Benito & Carmena, 2005) and good specificity (85-95%) (Benito and Carmena, 2005; Buishi *et al.*, 2005a; Allan and Craig, 2006). Although coproELISA benefits from being able to detect pre-patent infection, negative predictive value of the test can be affected by low worm burdens (<50-100 worms) (Allan & Craig, 2006).

There are several PCR protocols to detect *Echinococcus* spp. DNA (coproPCR) directly from faeces (Abbasi *et al.*, 2003; Boufana *et al.*, 2008, 2013; Lett, 2013) or eggs isolated from faeces (Boubaker *et al.*, 2013, 2016; Cabrera *et al.*, 2002; Štefanić *et al.*,

2004; Trachsel, Deplazes, & Mathis, 2007a). Although reported sensitivity and specificity can be high (100%) (Lahmar *et al.*, 2007), the technique can require costly equipment, laborious DNA extraction and can be affected by DNA inhibitors in faeces (Alexander Mathis & Deplazes, 2006; Trachsel, Deplazes, & Mathis, 2017). More recently, loop-mediated isothermal amplification (LAMP) methods have been developed, which rely less on specialized equipment (Salant, Abbasi, & Hamburger, 2012).

A serial approach of primary screening with a genus-specific coproELISA followed by testing positive samples with species-specific coproPCR has been recommended as a practical and cost-effective surveillance strategy (Craig *et al.*, 2015; Eckert *et al.*, 2002; Mathis, Deplazes, & Eckert, 1996). However, studies have found the correlation between the tests is not always optimal and can be affected by low worm burdens, coprophagia, pre-patent period infection and low egg counts (Craig *et al.*, 2015; van Kesteren, 2015). Therefore, testing of a proportion of negative samples or running the tests in parallel for all samples is advised (Craig *et al.*, 2015).

From a control perspective, the main target for intervention is the definitive canine host, with the aims to prevent infection, reduce adult worm burdens and reduce environmental contamination with faeces (Craig *et al.*, 2017; Otero-Abad & Torgerson, 2013; Palmer *et al.*, 1996b). This requires a multifactorial approach (Deplazes *et al.*, 2011; Craig *et al.*, 2017). Specific measures include preventing access to and feeding of raw meat and offal; risk-based worming with a praziquantel containing wormer i.e. a minimum of 4 times annually, increasing to 6-weekly if there is the risk from feeding or accessing raw offal and collection and disposal of faeces from the environment (WHO, 2002; Craig *et al.*, 2017; Vuitton, Zhang and Giraudoux, 2017).

Praziquantel, a pyrazinoisoquinoline derivative, is a widely-available wormer licensed for veterinary treatment and prevention of *Echinococcus* spp. in dogs and cats (National Office of Animal Health, 2017). The half-life of oral praziquantel is 3 hours, meaning there is little residual action against reinfection (EFSA Panel on Animal Health and Welfare, 2015). An oral dose of 5mg/kg, subcutaneous dose of 5.8mg/kg

and spot on dose of 12mg/kg (cats only) is effective against *Echinococcus* spp. (both adult and immature forms) (Vercruysse and Claerebout, 2019). Epsiprantel, a closely related analogue of praziquantel is also an effective and licensed cestocidal drug in companion animals, and effective against *Echinococcus* spp. at a dose of 5mg/kg, though is not currently widely available in the UK. (Vercruysse and Claerebout, 2019).

Although the role of pet dogs in the transmission of *Echinococcus spp*. has not been explored within the scope of the thesis, pet dogs play an important role in the transmission of zoonotic *Echinococcus spp.* most notably *E. multilocularis* (Eckert and Deplazes, 2004; Sager *et al.*, 2006; Deplazes *et al.*, 2011). At the time of writing, dogs entering the UK under the EU Pet Passport Scheme require worming with a praziquantel wormer within 5 days of UK entry, to prevent the risk of importing *E. multilocularis*, a notifiable disease in the UK. The risk of importing *E. multilocularis* via the increasingly popular practice of rehoming rescue dogs from overseas has been a cause for concern (Trees, 2017). The risk of raw food feeding of pet dogs and anthelmintic prescribing patterns in veterinary practice also present areas of important research from the perspective of E*. granulosus* transmission.

1.3.3.2 CE in the intermediate livestock host

The disease, diagnosis and detection of cystic echinococcosis in livestock is discussed in detail in Chapter 6. In brief, cysts are typically found in the liver and lungs, though can occur in other organs, such as muscle and bone. *Post-mortem*, hydatid cysts can reduce the overall value of a carcase through condemnation of affected organs, poor carcase weight and meat yield and in severe cases, rejection of the whole carcase when hydatid disease has led to emaciation of the animal (AHDB, 2018b).

Hydatid cysts in horses are diagnosed at slaughter or post mortem examination and there is little evidence that morbidity results from infection, despite cysts in the liver and lung predilection sites reaching a considerable size (Rezabek, Giles, & Lyons, 1993). Although it is broadly accepted that *E. equinus* is not infective to humans (McManus *et al.*, 1989; Moro & Schantz, 2009b; Romig *et al.*, 2006) definitive

conclusions cannot be drawn without extensive strain typing of a large number of human cystic echinococcosis cases (Eckert & Deplazes, 2004).

The main method for diagnosis is cyst detection during livestock meat inspection or at *post-mortem* examination of wild or captive intermediate hosts (Craig *et al*., 2015; Eckert & Deplazes, 2004). Cyst detection during necropsy is the reference gold standard for diagnosis, but the process requires thinly slicing the organ to locate cysts and examine cyst contents, and if possible histopathology and molecular confirmatory diagnosis (Eckert et al., 2002). Although this has been shown to increase the sensitivity and specificity of necropsy diagnosis during surveillance (Lloyd, Walters and Craig, 1998) it is not a logistically feasible process during routine meat inspection (Wilson *et al.*, 2019). *Ante-mortem* diagnostic methods assessed in livestock include ultrasound (Sage *et al.*, 1998; Lahmar *et al.*, 2007; Dore *et al.*, 2014) and serodiagnosis (Blundell-Hasell, 1969; Ibrahim, 2010), with varied success and challenges due to co-infection with other taeniid cestodes.

The main infection risk to domestic and wild intermediate hosts is grazing on pasture or feed contaminated with eggs voided in infected canid faeces (McManus *et al*., 1989). Age has been identified as a significant determinant of CE risk in livestock, with older animals recording higher CE prevalence (Banks *et al.* 2012) and increased cyst abundance (Ibrahim, 2010).

Hydatid lesions and affected organs in livestock at slaughter are removed during routine meat hygiene inspection in the UK and disposed of as category 2 animal by product (ABP) in accordance with Food Standards Agency (FSA) guidelines (FSA, 2018b). The risks of *E. granulosus* and *E. equinus* transmission associated with the feeding of ABP to dogs are covered in the three canine study chapters (Chapters 3,4 and 5). Cases of hydatidosis identified at slaughter are recorded on the FSA Collection and Communication of Inspection Results (CCIR) system. The resultant data are reported back to the farmer to inform any necessary disease control measures (AHDB, 2018b; FSA, 2018b).

1.4 The HyData project and the rationale for investigating echinococcosis in the UK

The name of the project, HyData, is a portmanteau of the words 'Hydatid' and 'Data'. The name was chosen to reflect the overarching aims of the thesis to update our understanding of recent trends in the burden of echinococcosis in UK animal populations and explore associated risk factors. The thesis investigates echinococcosis through four distinct studies and populations: hunting hounds, farm dogs, zoo canids and livestock. The current understanding of the epidemiology of echinococcosis in the UK is described from different perspectives through each of the four study chapters. Each also describes the rationale for undertaking the research in the way it did and why it was important to do so. The rationale and overarching aims of the thesis, with reference to echinococcosis in the UK, relate to three key hypotheses:

1. E. granulosus is a suspected re-emergent pathogen in endemic areas where control programmes have previously been instituted.

E. granulosus and *E. equinus* are known to occur in the UK and have been reported in humans and animals for many decades. *E. granulosus* was thought to be confined to areas of Mid-Wales, the surrounding English borders and the Western Isles of Scotland. Between 1974-1983, average annual incidence of human CE in Wales was 0.4 case per 100,000 people, rising to 7 cases per 100,000 people in Powys and Brecknockshire regions, compared with 0.02 cases in England (Palmer & Biffin, 1987). A voluntary hydatid control programmes in the 1970's-80's involving supervised worming of farm dogs successfully reduced hydatidosis levels by 90% in dogs and 50% in sheep (Palmer *et al.*, 1996) and by the early 1990's hospital admissions of human CE cases had dropped to negligible levels in the intervention areas (Palmer *et al.*, 1996). The programme in Wales was terminated prematurely due to limited funds and was replaced by a health education programme. Since the programme end, coproantigen prevalence in farm dogs has steadily risen in previous intervention areas (Buishi *et al.*, 2005b), with the most recent study reporting 10.6% at the individual dog level and 12.3% at the farm level (Mastin *et al.*, 2011). A retrospective

coproPCR study of samples from farms in the Welsh counties of Powys and Wales collected during these previous coproantigen-based studies (Buishi *et al.*, 2005a; Mastin *et al.*, 2011) reported 15.0% of dogs had *E. equinus* DNA in their faeces and 85.0% had *E. granulosus* DNA (Boufana *et al.*, 2015). The effect of this increased prevalence in endemic areas on public health is not known. Because of the long incubation period of the parasite, people infected may not show clinical signs for several decades (Moro & Schantz, 2009a). The re-emergence of *E. granulosus* in endemic areas was recently described as a potential 'ticking time bomb of hydatid disease' by the director of Public Health England's parasitology reference laboratory (Anon, 2017). Evidence of ongoing *E. granulosus* transmission in endemic areas of Wales has been supported by coproantigen and more recently, coproPCR studies, of hunting packs in the endemic region (Lett *et al.*, 2018; Thompson & Smyth, 1975). Since 2008 and 2011 respectively, no further studies have investigated this strong trend of increasing prevalence in farm or hunting dogs.

The farm dog study (**Chapter 5**) aims to undertake the first UK-wide investigation of echinococcosis at the genus and species level in this population. This will not only build on recent molecular evidence of the re-emergent picture of disease in Mid-Wales, but also to areas bordering this region and beyond. The hunting hound study (**Chapter 3**) will undertake a UK-wide study of *Echinococcus* spp. infection in hunts operating both within and outside known endemic regions. Overall, the studies will contribute to the understanding of renewed *E. granulosus* transmission in the UK in order to better inform surveillance, public health information and future control efforts.

2. Preliminary evidence appears to support disease spread beyond known areas of high E. granulosus prevalence.

The majority of human CE cases identified in the UK have a history of travel to highly-endemic areas and are likely to have been contracted overseas (Deplazes *et al.*, 2017). Historical non-imported cases relating to the period of high endemicity are confined to Wales and the Welsh border regions (Palmer & Biffin, 1987; Stallbaumer, 1987). More recent case report evidence of autochthonous CE in a foxhound worker

in the Southwest of England (Craig *et al.*, 2012), an engineer in Cumbria (Boufana *et al.*, 2015) and a visitor from a non-endemic country to England (Hibiya *et al.*, 2011), suggest a public health risk beyond known areas of *E. granulosus* endemicity.

A possible wider transmission of *E. granulosus* beyond known endemic areas is also supported by the finding of two coproantigen and coproDNA positive hunting packs in Northumberland (Lett *et al.*, 2018).

A recent pilot study, utilizing hydatid data from cattle raised throughout the UK as sentinel hosts, has provided strong evidence that the parasite is more widely distributed than previously thought (Temple, Jones and Brouwer, 2013). Analysis of recorded cyst material in condemned offal during routine inspection of cattle at slaughter in 19 abattoirs in Wales between 2010-2011, together with cattle movement records, indicate that areas of highest prevalence remain in mid-Wales. Although not representative for England and Scotland as a whole, further hotspots existed in the midlands of England, Manchester and Perthshire in Scotland (Temple, Jones and Brouwer, 2013).

Based on these case report and pilot study data, there is an urgent need for further investigation of *E. granulosus* beyond historically prevalent areas, in particular using molecular methods to identify the parasite to species level (**Chapter 2**) (Boufana *et al.*, 2015; Lett *et al.*, 2018; Mastin *et al.*, 2011). All four UK-wide studies within the thesis aim to test the hypothesis that *E. granulosus* transmission is occurring in regions understood to be non-endemic for the parasite. Using molecular testing of hydatid cysts identified at slaughter in cattle throughout the UK, together with their movement records, the abattoir study (**Chapter 6**) aims to detect *the parasite* to species level, in particular in animals that have never travelled within known endemic areas. The three canine studies aim to detect *E. granulosus* within hunt, zoo and farm dog populations to build a picture of infection distribution at the national level; to the author's knowledge, the first to do so using molecular diagnostic methods. Furthermore, the farm dog study (**Chapter 3**) aims to undertake a freedom from disease analysis at a regional UK level. Resultant data aims to contribute to testing the overarching thesis hypothesis that *E. granulosus* in definitive canine and

intermediate livestock hosts in the UK is not confined to historic hotspots in Wales and the Hebridean Islands.

3. There are a-priori and putative risk factors for infection in definitive and intermediate hosts and their environment that have not been sufficiently explored in the UK.

There are many recognised risk factors associated with canine infection of *E. granulosus* and *E. equinus*, and these are described in section 1.5.1 of this introduction. The study populations of the three canine studies are definitive hosts of *E. granulosus* and *E. equinus* with evidence of potential routes of *Echinococcus* spp. transmission in their respective environments. Through contact with these canines populations and their faeces, farmers, hunt workers, zoo keepers and veterinarians are at greater risk of occupationally-acquired CE (Health and Safety Executive, 2015).

The three canine studies in foxhounds, farm dogs and zoo canids focus on the commonality of a number of key transmission factors for *E. granulosus* and *E. equinus;* these include access to high-risk raw meat and offal products either by regulated or unregulated feeding of animal by products from the slaughter industry or fallen stock and opportunity to scavenge infected carcasses (Moro and Schantz, 2009a; Otero-Abad and Torgerson, 2013; Acosta-Jamett *et al.*, 2014; Romig *et al.*, 2017).

Transmission cycles are maintained in many wild definitive carnivore and intermediate host species (Otero-Abad & Torgerson, 2013; Romig *et al.*, 2015) yet very little is known about the transmission of *Echinococcus* within such species in zoo collections (Boufana *et al.*, 2012). Fatal cases of *E. equinus* and *E. granulosus* in captive-bred zoo mammals in UK zoo collections have been reported, but the route of infection in such cases is unknown (Boufana *et al.*, 2012; Denk *et al.* 2016, . To the authors knowledge, the risks of *Echinococcus* spp. transmission arising from the shared environment between captive canine hosts and other animal species and zoo personnel have not been explored. The zoo study represents the first UK-wide investigation of *Echinococcus spp.* in canid and hyaenid zoo collections. The study

aims to identify the potential risks of *Echinococcus* spp. transmission arising from the specialised animal husbandry practices in this setting (**Chapter 4**).

Sheep farmers, zoos and hunting packs are represented by stakeholder associations that protect the interests of their members and promote best practice. The National Sheep Association (NSA), the British and Irish Association of Zoos and Aquariums (BIAZA) and the Council of Hunting Associations (CHA) are all collaborators in the HyData project. Regular worming, collection and disposal of faeces and biosecurity measures are all recommended under health and husbandry guidelines issued to the study groups (farms, zoos and hunts) through their representative associations (BIAZA, 2014; NSA, 2019a; The Council of Hunting Associations, 2015). However, research to better understand whether such measures adequately address the risks to animals and humans from zoonoses such as *E. granulosus* in these settings is lacking. Evidence suggests that anthelmintic use in high-risk dog populations is suboptimal (Lett, 2013). A key objective of the thesis is to explore and evidence shortcomings in de-worming dosing practices in these high-risk canine populations in the UK, as a basis to inform on optimal and targeted treatment protocols.

The role that other husbandry-associated risk factors, such as adequate disposal of faeces, play in the emergent and re-emergent picture of echinococcosis in the UK require urgent investigation. Through a questionnaire and faecal testing approach, the canine studies aim to understand the extent to which practices that are potentially important to the transmission dynamics of *Echinococcus spp*. take place use the findings to inform current practices designed to mitigate the risk of transmission.

Chapter Two

Methodology for canine HyData studies

2 Methodology for Canine HyData studies

2.1 Introduction

The thesis comprised four separate observational cross-sectional surveys of animal populations of interest to the epidemiology of echinococcosis and hydatid disease in the UK: hunting hounds, zoo canids and hyaenids, farm dogs and livestock (cattle and sheep). The studies involved collaboration with national associations representing each of the animal populations of interest and those who work with them. Each involved a reciprocal arrangement whereby the professional association assisted with the recruitment of participants in return for an anonymised report of the findings. The data returned were of interest to the professional organization services and aimed to establish points of discussion based on the study findings. In each case, the professional association was involved in representing, monitoring, inspecting and regulating the actions of hunts, farms, zoos and abattoirs respectively, in relation to public health, animal health and welfare. This chapter describes the materials and methods common to the studies in hunt packs, zoos and farm dogs. Any individual variations in study design, external collaborator and laboratory protocols in each are covered in detail in the relevant study chapters.

2.2 Study design

The three canine studies were designed as prospective cross-sectional surveys of *Echinococcus* spp. in domestic and captive wild canine populations in the UK. The studies employed a questionnaire and canine faecal sampling approach to be undertaken by hunt staff, zoo staff and farmers respectively and returned by post to the researcher. In each case, the relevant collaborating associations; the Hunting Office, BIAZA and the NSA assisted by informing members about the study and endorsing the work. The NSA also assisted in the anonymization and recruitment of member farms.

A convenience sampling approach using open source member databases was used to obtain the contact details of study participants in the hunt and zoo studies, which had relatively small numbers of premises. Recruitment was via email, postal

invitation, telephone call or a combination, depending on the contact details available. The farm dog study, a much larger population, used an anonymized member list provided by the NSA and a randomization step (detailed in Chapter 5) was introduced to select member farms from UK regions. The NSA contacted selected farms via mail or email to invite participation, and those wishing to do so contacted the author directly. At the end of the research, it was agreed that collaborators would receive an anonymized summary of the study results. Individual study results were made available to participants of the zoo and farm dog studies on request, though not in the hunt study for the reasons discussed in Chapter 3.

The canine studies were based on the collection of pooled samples, rather than individual faecal samples. As voided faeces were being collected by the respondents, often from a kennel or enclosure environment, it would not have been practical or reliable to pursue the identity of individual animals. As such, the study units were the hunt, the single-species zoo enclosure and the farm. The methods used for the detection of *Echinococcus* spp. in faeces were a genus-specific coproantigen ELISA (coproELISA) and four PCR protocols: a genus-specific multiplex cestode PCR, and species-specific PCR protocols for *E. granulosus* sensu lato, *E. granulosus* G1 (sheep strain) and *E. equinus*.

2.3 Ethical approval

Individual applications for each of the HyData studies were submitted to the University of Liverpool Research Ethics Committee and approvals granted under the expedited review process for the Institute of Infection and Global Health, University of Liverpool. Further details are provided in the relevant chapters.

2.4 Questionnaire design

For each study group, a participant questionnaire was designed to test the hypothesis that risk factors relating to husbandry (diet, sites of exercise, hygiene practices) and endoparasite control (worming treatment and parasite testing where relevant) were present and relevant to *Echinococcus* spp. infection. The overall prescribed response format of the questionnaire was similar across the three studies

though some lines of questioning were specific to the individual study settings. Examples of each questionnaire, accompanying participant information sheets in a Frequently Asked Questions (FAQ) format are included in Annex for each chapter.

Prior to circulation, draft questionnaires were reviewed by a panel of four academic peers at the University of Liverpool familiar with questionnaire design for cross-sectional studies. This measure provided objective guidance on ensuring that questions were concise, unambiguous and related directly to the testing of hypotheses within the associated study. Individual study draft questionnaires were also appraised by a representative of the relevant collaborating institution to ensure wording and context were correct for the setting and survey participant group. Question format was mainly closed or occasionally closed with open-response option to facilitate consistent classification and analyses of the data (FAO, 1997).

2.5 Sampling kit design

The thesis used postal questionnaires and sample collection and assessed the feasibility of this approach in the study settings. Pooled faecal sampling and analysis at the hunt, zoo enclosure and farm level were used to test the hypotheses that *E. granulosus* is a re-emergent parasite in areas where it has previously been controlled; that transmission is occuring beyond these known areas and that infection relates to *a-priori* and putative risk factors. A sample kit was prepared for each participant and the content and format of the kit for each of the three canine studies was the same. Each sampling kit included two 20ml sterile screw top faecal sample pots with inbuilt spatula placed within Specisafe® biological sample mailing packs (Alpha Laboratories, UK) for the collection of fresh voided faeces, latex-free disposable gloves, participant information sheet, questionnaire, step-by-step sample collection and packaging guide, consent form and pre-paid First-Class Royal Mail envelope for return of post (Figure 2-1).

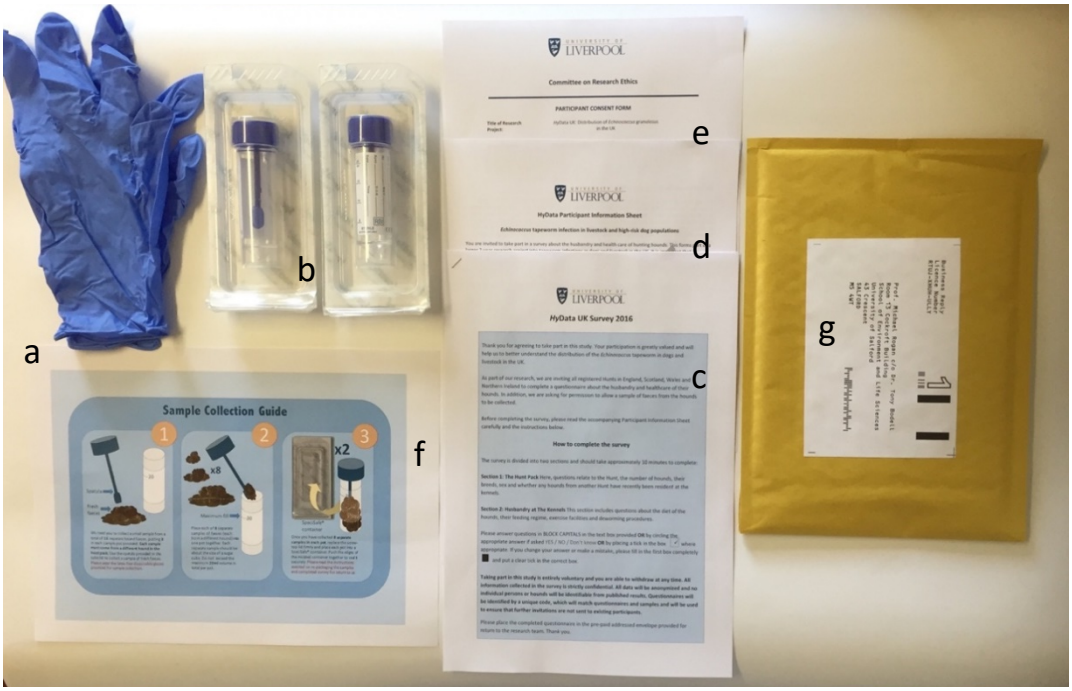

Figure 2-1. Sampling kit for the HyData canine studies each containing (a) latex-free gloves, (b) two faecal sample pots within Specisafe® biological sample mailing packs, (c) questionnaire, (d) participant information sheet, (e) participant consent form, (f) step-by-step sample collection instructions and pre-paid self-addressed return envelope (g).

For transport purposes, faecal samples collected for the project were classified as Category B Biological Substances, which are assigned to UN3373 Sample Transport Compliance Regulations (Department for Transport, 2012). Sample containers and mailing packaging were UN3373 compliant and packaged in accordance with International Carriage of Dangerous Goods by road (ADR) Packing Instruction 650 (PI650) regulations. An individual sample package was permitted to hold a total maximum of 50ml of liquid or 50g of solid faecal material. (Department for Transport, 2012).

Participants were requested to collect individual (if there was only one dog at the premises) or pooled voided faeces on a single occasion as per instructions for each study (see individual study chapters) and advised to complete the questionnaire at the time of sample collection. The importance of returning the questionnaire and fresh faecal samples by Royal Mail post as soon as possible was emphasised.

Risk assessments for all stages of sample collection and laboratory work were undertaken in accordance with current Control of Substances Hazardous to Health (COSHH) requirements. Under COSHH regulations, *E. granulosus* is classified as a

Hazard Group 3 infectious agent typically requiring Containment Level 3 measures (CL3) to undertake diagnostic work (HSE, 2013). However, as the diagnostic work undertaken had no intention to propagate or concentrate the viable agent, it was permissible under the regulations to undertake the work within Containment Level 2 (CL2) measures, with additional steps to protect against skin penetration injury and ingestion (HSE, 2013). Sample processing and laboratory testing were conducted at Leahurst Veterinary Campus, Wirral or the University of Salford, Greater Manchester. On arrival, faecal samples were frozen at -80˚C for a minimum period of one week to destroy any viable *Echinococcus* spp. eggs before sample processing (Eckert *et al.*, 2002) and then stored at -20˚C until further analysis.

2.6 Coproantigen ELISA (coproELISA)

2.6.1 CoproELISA reagent and cut off value selection

A polyclonal genus-specific coproELISA for detection of *Echinococcus* spp. antigen from adult worms or eggs present in faeces was used for all the canine studies. The ELISA used a plate-immobilised capture antibody to bind sample antigen, which was then detected by a second enzyme-conjugated antibody binding to a different epitope on the antigen. All coproELISA work was undertaken by the author at the Cestode Zoonoses Research Laboratory, University of Salford, using a protocol based on the methods of Allan *et al.* (1991) and Craig *et al.* (1995) further optimised at the University of Salford by van Kesteren *et al.* (2013).

The polyclonal capture (R5) and conjugate (R91) IgG antibodies used for the coproELISA were provided by Prof. Michael Rogan at the University of Salford. These antibodies had been previously isolated from sera of rabbits immunized with *E. granulosus* whole worm extract or a saline wash of intact *E. granulosus* worms as part of echinococcosis research undertaken in Northwest China (Feng, 2012). R91 antibodies had been conjugated with horseradish peroxidase enzyme and stored at -80˚C until use (van Kesteren *et al.*, 2013).

An R5 (1:1500) capture and R91 (1:1500) conjugate antibody concentration combination had previously been selected as giving optimal signal:noise ratio by

chequerboard titration and tested against a panel of known negative and positive *Echinococcus* canine faecal samples, reporting a sensitivity of 93.5% and specificity of 100% (van Kesteren, 2015). This combination was in routine use for validated commercial coproantigen diagnostics at the University of Salford (van Kesteren *et al.*, 2013) and was used for all assays undertaken for the thesis.

The cut-off value for a positive/negative threshold was determined by the Gaussian approach; a method that assesses the clinical value and cut-off of the test based on a value that will correctly classify most negative samples (Allan *et al.*, 1992; Deplazes *et al.*, 1992) on the assumption that the OD of a panel of negative samples will follow a Gaussian distribution. Under such conditions, the approach should correctly classify 99.9% of all negative samples i.e. a specificity of 99.9%. The cut-off value was calculated as equal or greater than three standard deviations (SD) above the mean optical density value for a panel of negative sample. This panel comprised 48 canine faecal samples from the Falkland Islands, classed as a very low-endemic area, collected for separate ongoing research at the University of Salford. All dogs residing in the Falkland Islands are registered and wormed with a praziquantel product every 4 weeks as part of a longstanding Government-managed dosing programme (Craig *et al.,* 2017). This negative control panel was selected as a reliable and comparable sample panel for establishing a test cut off value for testing UK-based canine faecal samples in a similar very low-endemic setting.

Faeces spiked with *E. granulosus* whole worm extract and previously collected samples of known positive infection i.e. *Echinococcus* spp. infection confirmed by arecoline purging or necropsy from studies in Kyrgyzstan and China undertaken by researchers at the University of Salford (van Kesteren *et al.*, 2013, 2015) were used as positive and proxy positive controls. Samples from dogs naturally infected with *E. equinus* were not available to this study. A known negative faeces sample was spiked with cyst fluid from a UK horse hydatid cyst, sourced from a knacker's premises in the North West of England, for use as a proxy positive control.

2.6.2 CoproELISA method

Immulon 4HBX flat-bottom 96-well plates (ThermoFisher Scientific, UK) were coated with 100µl of R5 capture antibody (1:1500) diluted in Carbonate Bicarbonate Buffer (Sigma Aldrich, UK) (except blank wells), covered and incubated overnight at 4˚C.

The following day, plates were washed three times with 0.1% Phosphate Buffered Saline with Tween 20™ (PBST) (Sigma Aldrich, UK). As a blocking step, 100µl of 0.3% PBST was added to wells (except blank wells) and plates were incubated for 1 hour at room temperature then contents discarded and the plates patted dry.

Individual and pooled faecal samples were thawed and 3g of faeces per sample suspended in 10ml of 0.3% PBST. The suspension was vortexed for 1 minute until fully homogenized and centrifuged at 2500 r.p.m (1125G) for 5 minutes in an Eppendorf Centrifuge 5804 (Eppendorf UK Ltd., UK). The resultant supernatant was collected and used as the test sample for the coproELISA. All samples and controls were analysed in triplicate.

To each well, 50µl of test faecal supernatant and 50µl of foetal bovine serum (ThermoFisher Scientific, UK) were added and gently mixed by pipetting. Plates were incubated for 1 hour at room temperature then contents discarded and plates washed three times with 0.1% PBST. To each well (except blanks) 100µl of R91 conjugate antibody (1:1500) diluted in 0.3% PBST was added and plates incubated for 1 hour at room temperature. Following a further three washes with 0.1% PBST, 100µl of SureBlue® TMB substrate (Sigma Aldrich, UK) was added to all wells and plates were incubated in the dark for 20 minutes. Plates were read using a 620nm filter on a Multiskan FC plate reader (ThermoScientific, UK).

The average of the blank-subtracted triplicate test sample OD values was used as the result for that sample. Failure of positive and/or negative controls i.e. OD values not respectively above or below the predetermined cut-off value prompted a repeat of the whole plate assay. Sample replicates giving OD values above and below the cut-off point prompted a repeat of the test for the sample with four replicates instead of

three. Results were stored on a designated Microsoft Excel spreadsheet for each study (Microsoft Corporation, Redmond, Washington, USA).

2.7 Coproantigen Polymerase Chain Reaction (coproPCR)

2.7.1 DNA extraction

Genomic DNA (coproDNA) was extracted from thawed canine faecal samples using a QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol including advised modifications for isolation of DNA from larger volumes of stool for downstream pathogen detection. This modified protocol was recommended when target DNA is at low concentration and/or may not be distributed homogenously within the starting material (QIAGEN, 2014). Modifications included a starting volume of 1-2g of faeces per sample (depending on amount of sample available) and addition of an equivalent 10-fold volume of InhibitEX buffer. A subsequent lysis step was performed at a higher temperature of 95˚C (conventionally 70˚C), recommended for target cells of parasite origin that may be difficult to lyse (QIAGEN, 2014). Purified, concentrated eluted DNA in a 200µl volume of low-salt buffer was stored at -20˚C until further use. DNA concentration was determined by absorbance at 260nm and purity by the A260/A280 ratio of absorbance using a Nanodrop™ spectrophotometer (Thermo Scientific, UK).

2.7.2 PCR controls: Template tissue and faecal samples of known infection status

Extracted DNA from adult worms of *E. granulosus* G1, *Taenia hydatigena*, *Taenia ovis*, *Taenia multiceps*, *Taenia pissiformis* and *Dipylidium caninum,* collected and sequenced for use in diagnostic testing at the University of Salford were donated by Professor Michael Rogan and used as template controls.

E. equinus DNA was obtained from whole cyst material from the liver of a horse donated to the author from a local knackers yard in the North West of England. Cyst fluid and cyst lining were DNA extracted using Qiagen DNeasy® Blood and Tissue kits according to manufacturer's instructions (QIAGEN, 2006). Identity was confirmed by PCR amplification of a ~450bp fragment of the mitochondrial cytochrome c oxidase

subunit 1 (*cox*1) (Bowles, Blair and McManus, 1992), sequencing of the amplicon (Source BioScience, UK) and a 100% match against *E. equinus* mitochondrial DNA (accession number EF143835.1) via a BLAST search of the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

DNA extracts from fresh voided faeces of three urban UK pet dogs regularly wormed with a praziquantel-containing product according to manufacturer's instructions, with the last worming dose 7 days previously, were used as additional negative controls for PCR protocols. DNA extracts from three faecal samples of known positive *E. granulosus* infection (confirmed by arecoline purgation) collected during a previous research study in Kyrgyzstan and shown to give a positive coproELISA and coproPCR result (van Kesteren *et al.*, 2013) were used as positive controls. Further positive control samples were prepared by 'spiking' the negative control UK faecal samples with whole worm extract from *E. granulosus* G1 and known numbers of *E. granulosus G1* eggs (provided by Professor Michael Rogan, University of Salford).

2.7.3 CoproPCR protocols

There are several published PCR protocols for the amplification and detection of *E. granulosus* DNA in faeces (Cabrera *et al.,* 2002; Abbasi *et al.,* 2003; Dinkel *et al.,* 2004; Trachsel, Deplazes and Mathis, 2007; Boubaker *et al.,* 2013) and more specifically genotypes within the *E. granulosus* complex (Štefanić *et al.,* 2004; Boufana *et al.,* 2013; Santucciu *et al.,* 2019). Four PCR protocols with increasing specificity of detection were selected for the canine studies. The coproPCR work flow was designed to first identify and differentiate *Taenia* spp. and *Echinococcus* spp. coproDNA (Trachsel et al., 2007b) then detect *E. granulosus* (Abbasi *et al.*, 2003) and finally detect *E. granulosus* G1 (sheep strain) (Boufana *et al.*, 2013) and *E. equinus* (Lett *et al.*, 2018).

All PCR reactions were performed in an Applied Biosystems 2720 Thermocycler (Applied Biosystems, UK). PCR grade water (Sigma Aldrich, UK) at equal volume to template DNA was included in every reaction as a non-template control. All PCR primers were synthesized by Eurofins (Eurofins MWG Operon, Germany). PCR

products were resolved at 110V on a 1.5% or 3% (w/v) Agarose gel (Biogene Ltd., UK) in 1x Tris-Acetate-EDTA buffer (Sigma-Aldrich, UK) stained with PeqGREEN dye (VRW Peqlab, USA). Fragment band size was estimated by comparison with a 100bp DNA ladder (Solis Biodyne, UK) or a Low Molecular Weight DNA Ladder (New England Biolabs inc, USA) depending on anticipated amplicon size(s). Gels were visualized on a UVITEC Gel Documentation System (UVITEC, UK) using UVIProMV v11.02 software (UVITEC, UK).

2.7.3.1 Multiplex PCR protocol

The multiplex protocol developed by Trachsel, Deplazes and Mathis (2007) amplifies a 267bp fragment of small subunit ribosomal DNA (*rrn*S) from *Taenia*, *Mesocestoides*, *Dipylidium* and *Diphyllobothrium* spp; a 117bp *rrn*S fragment from *E. granulosus* and a 395bp NADH dehydrogenase subunit 1 (*nad*1) fragment from *E. multilocularis* mitochondrial gene. The authors report an analytic sensitivity of 1 taenid egg per gram of faeces (Trachsel et al., 2007b).

The protocol was modified to include only primer pair selections amplifying the 117bp *E. granulosus* DNA fragment (*Cest 3* and *Cest 5*) and the 267bp Taenid DNA fragment (*Cest 4* and *Cest 5*). The primer pair (*Cest 1* and *Cest 2*) designated to amplify the 395bp DNA fragment from *E. multilocularis* was omitted as this cestode species is not reported in the UK and its research falls beyond the scope of this study. PCR reactions were conducted in a total reaction of 40µl comprising 10µl of template DNA prepared with 5x FirePol® Ready to Use Master Mix (Solis Biodyne, Tartu, Estonia) containing 7.5mM $MgCl_2$, 100pmol of forward primers *Cest 3*, 5'-YGAYTCTTTTTAGGGGAAGGTGTG-3' and *Cest 4*, 5'- GTTTTTGTGTGTTACATTAATA AGGGTG-3' and 200pmol of the shared reverse primer *Cest 5*, 5'-GCGGTGTGTA CMTGAGCTAAAC-3' (suspended in 1µl of PCR grade water) supplemented with PCR grade water to a total 40µl reaction volume. Thermal cycling conditions were as follows: an initial denaturation step of 5 minutes at 95˚C, followed by 30 cycles of 30 seconds at 95˚C, 1 minute at 56˚C and 40 seconds at 72˚C and a final elongation step of 10 minutes at 72˚C (Trachsel *et al.*, 2007b).

2.7.3.2 PCR protocol for amplification of E. granulosus

The protocol developed by Abbasi *et al.* (2003) amplifies a 269bp segment within the EgG1 *Hae* III tandem repeat unit identified in the genome of *E. granulosus* G1 (sheep strain) (Abbasi *et al.*, 2003). Further evaluation of this protocol by Boufana *et al.,* (2008) found the protocol unable to reliably differentiate between the genotypes of *E. granulosus*. However, the protocol remained highly species specific, with analytical sensitivity to detect a single egg, therefore remains useful in the confirmation of *E. granulosus* infection in dogs (Boufana *et al.*, 2008; Craig *et al.*, 2015).

The protocol was modified to include the addition of 2% formamide to the reaction mix to enhance PCR amplification and improve reaction specificity (Boufana *et al.*, 2008). PCR reactions were conducted in a total reaction volume of 40µl comprising 10µl of template DNA. Reactions were prepared with 5x FirePol® Ready to Use Master Mix (Solis Biodyne) containing 7.5mM $MgCl_2$, 100pmol of each primer, Eg2691, 5'-ACACCACGCATGAGGATTAC-3' and Eg2692, 5'-ACCGAGCATTTGAAATGTTGC-3', suspended in 1µl of PCR grade water, supplemented with PCR grade water to a total 40µl reaction volume. Thermal cycling conditions were as follows: an initial denaturation step of 5 minutes at 95˚C, followed by 35 cycles of 1 minute at 95˚C, 1 minute at 55˚C and 1 minute at 72˚C and a final elongation step of 10 minutes at 72˚C (Abbasi *et al.*, 2003; Boufana *et al.*, 2008).

Genus-specific PCR Protocols for E. granulosus G1 and E. equinus

The *E. granulosus* G1 protocol developed by Boufana *et al.*, (2013) amplifies a species-specific 226bp segment within the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene. PCR reactions were conducted in a total reaction volume of 50µl comprising 1µl of template DNA. Reactions were prepared with 5x Flexi reaction buffer (Promega Ltd.) containing 2mM $MgCl_2$, 200mM of each deoxynucleoside triphosphate (dNTPs, Bioline), 2.5U GoTaq polymerase (Promega, Ltd.) 0.3mM of each primer, Eg1F81, 5'-GTTTTTGGCTGCCGCCAGAAC -3' and Eg1R83, 5'-AATTAATGGAAATAATAACAAACTTAATCAACAAT-3', supplemented with PCR grade water to a total 50µl reaction volume. Thermal cycling conditions were as follows: an initial denaturation step of 5 minutes at 94˚C, followed by 30 cycles of 30 seconds at

94˚C, 50 seconds at 62˚C, 30 seconds at 72˚C and a final elongation step of 5 minutes at 72˚C (Boufana *et al.*, 2013).

The *E. equinus* protocol developed by Lett (2013) amplifies a species-specific 299bp segment within the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene. The reaction volume and master mix components were the same as those detailed for the *E. granulosus* G1 protocol detailed above, except using 0.3mM of each primer, G4F, 5'-GGTTTTGAGATACATAATAATGTCCGGAC-3' and G4R, 3'-CTCACACCAAGCACCTACACATAAATATAGTT-5'. Thermal cycling conditions were as follows: an initial denaturation step of 5 minutes at 94˚C, followed by 35 cycles of 30 seconds at 94˚C, 30 seconds at 59˚C and 30 seconds at 72˚C with no final elongation step (Lett *et al.*, 2018).

The methodology included different post-DNA extraction steps where appropriate to mitigate the effects of PCR inhibitors, including addition of formamide (Boufana *et al.*, 2008; Chakrabarti & Schutt, 2001; Ito, 2013) or Bovine Serum Albumin (Ito, 2013; von Nickisch-Rosenegk, Silva-Gonzalez, & Lucius, 1999) to the PCR reaction mixture to purify template material prior to PCR. Furthermore, where test samples returned a negative PCR result, samples were diluted 1:10 and 1:100 in PCR grade water to dilute PCR inhibitors present and the reaction repeated. All PCR products were stored at -20˚C until further use.

Chapter Three

Echinococcus spp. in UK hunting hounds

3 *Echinococcus spp. in UK hunting hounds*

3.1 Introduction

E. granulosus and *E. equinus* are endemic to the UK (Craig *et al.*, 2017; Otero-Abad & Torgerson, 2013; Rinaldi *et al.*, 2017). *E. granulosus* transmits predominantly through a domestic dog-sheep lifecycle and can infect other livestock species and humans as accidental dead-end hosts (Craig *et al.*, 2015). Foxhounds packs have been identified as hosts for *E. granulosus* (Boufana *et al.*, 2015; Lett, 2013; Lett *et al.*, 2018; Thompson & Smyth, 1975; Williams, 1992) and are understood to be the main definitive host for *E. equinus* in the UK (Thompson & Smyth, 1975; Rinaldi *et al.*, 2017). Furthermore, a case of occupational zoonotic transmission in the UK has been linked to working with hunting packs (Craig *et al.*, 2012). This chapter describes a UK-wide survey of canine echinococcosis in hunting hound packs and the first to include different types of hunting packs active in the UK. Using questionnaire, genus-specific coproELISA and species-specific coproPCR methods, this study investigates the occurrence of *Echinococcus* spp. in UK hunts. It also explores the potential risk factors for infection and transmission and explores the implications of the findings for animal and human health.

3.1.1. Hunting with hounds in the UK

The UK has a longstanding, complex and contentious tradition of hunting with hounds. At the time of writing, the UK had 385 registered hunting packs, spanning a variety of hunting disciplines, hound breeds (dogs that hunt by scent) and quarry. Hunts are listed in Baily's Hunting Directory, a comprehensive and publicly available list of hunting packs (Bailys Hunting Directory, 2019).

The Council of Hunting Associations (CHA) represents fourteen hunt associations by hunt type and quarry in England, Scotland and Wales. The administrative centre of the CHA is The Hunting Office, which advises member hunts on matters relating to hunting activities, hunt management and the health and welfare of hounds (The Hunting Office, 2019). The largest of the UK hunt associations is the Masters of Foxhounds Association (MFHA) representing 178 packs that hunt in England, Wales

and Scotland (The Hunting Office, 2019). The Hunting Association of Ireland represents six constituent hunting associations and plays a similar role to the CHA (The Hunting Association of Ireland, 2018).

The main breeds of hound used for live quarry hunting are foxhound, beagles, harriers, fell hound, basset and mink hound. Other breeds such as drag hounds and bloodhounds almost exclusively hunt an artificial trail. The four main traditional types of live quarry were fox, deer, hare and mink. Legislation within England, Wales and Scotland now prohibit the intentional pursuit of these and other wild animals with one or more dogs, with certain exemptions (further detail on these and Hunting Act is provided in section 3.1.2). A day's hunting or 'hunt meet' typically involves pack-led pursuit of either quarry or a laid scent trail followed by hunt members on horseback or on foot. Hunt spectators (often members of the Hunt Supporter's Club) follow the hunt on foot or by car. Despite changes in the law that limit traditional hunting (see section 3.1.2), hunts maintain an active annual hunting calendar, with the season for hunt meets broadly spanning October until April, depending on the hunt type.

Each hunt has a specified 'hunt country', a geographical range over which it will hunt. Large areas of land are covered during a day's hunt, often over several adjoining rural landholdings, with consent of the landowners. Prior to The Hunting Act (2004), hunts operated in around 70% of rural land in England and Wales, with an average of 29% of land not hunted due to denied access or safety concerns (Great Britain, 2004; Harris & Dorning, 2017). Within Scotland, hunting country is largely focused around the border regions, due to the availability of suitable terrain. It is common practice for a hunt to visit another hunt's country by invitation, a practice called a 'sporting visit' and for hunts to host hounds from a visiting pack at their kennel.

3.1.2. UK legislation relating to hunting with hounds

The Burns Inquiry was established by the UK Government in 1999 as a committee of inquiry into hunting with dogs in England and Wales (Burns, 2000). The Inquiry published its report in June 2000, and following a series of parliamentary votes, an

amended bill to ban hunting with dogs was introduced in September 2004 and was enacted into law as the Hunting Act (2004).

The Hunting Act (2004) came into force in February 2005 to control the hunting of wild animals with dogs and prohibit hare coursing in England and Wales. Although the act bans the pursuit of live quarry with a pack of dogs, there are several exemptions within Schedule 1 of the Hunting Act (2004). For example, a pack of hounds pursuing live quarry must be called off to allow the stalking and flushing out of the quarry with a maximum of two hounds to then be shot by a marksman (Great Britain., 2004). Hunting with dogs in Scotland is controlled by the Protection of Wild Mammals (Scotland) Act (Scottish Government., 2002). The law in Scotland permits the flushing out of foxes from cover by any number of hounds to then be shot by a marksman, although in January 2019, Scottish ministers pledged to limit this activity to a maximum of two hounds, bringing legal activity in line with the law in England. Ministers further pledged to ban all 'trail hunting' north of the border (Scottish Government, 2019). Ministers have also indicated an intention to implement a number of recommendations issued in the 2016 Bonomy report (Bonomy, 2016), a review of hunting legislation under the Protection of Wild Mammals (Scotland) Act (2002), which includes recommendations on licencing of hunts, developing a code of practice for hunts and strengthening of prosecutions for breaks in the law. At the time of writing, hunting with hounds remains legal in Northern Ireland with no restrictions.

Despite the hunting ban, the majority of hunts in the UK remain active in the field, pledging to hunt within the law, to exercise horses and hounds or to hunt an artificial scent trail instead of live quarry where permitted (Macdonald & Johnson, 2015). The social function of the hunts continues to play a part in the country calendar of many rural parts of the UK. Satellite outdoor events in the hunting calendar involving the hunt pack include point-to-point races, puppy shows, charity events, hunter trials and regional agricultural shows (The Hunting Office, 2019).

3.1.3. Biosecurity and hunting with hounds

3.1.3.1. General biosecurity and disease risks

A hunt meet can involve a large pack of hounds, riders on horseback, followers on foot and vehicles travelling over large areas of countryside. Long distances can be covered between farms, over agricultural and horticultural land, waterways and public rights of way. Such activities can be at odds with biosecurity measures recommended to livestock farmers to mitigate the risk of livestock disease transmission (Harris & Dorning, 2017). Biosecurity measures in UK guidelines on disease prevention for livestock farmers consider disease transmission via fomites i.e. materials and objects that can transfer pathogens between locations, particularly via soil or faecal contamination. Such measures include cleaning and disinfection of vehicles and equipment before travel on and off the farm; provision of washing and disinfection facilities for visitor's and worker's boots, hands and equipment and proper storage and collection facilities for fallen stock (Great Britain, DEFRA, 2012; Northern Ireland Government, DAERA, 2004; Scottish Government, 2014).

Dog fouling presents a direct disease transmission risk to animals and humans (Harris & Dorning, 2018; Lowe *et al.*, 2014). Dog fouling is legislated by the devolved administrations and controls fouling in public spaces, however it includes no legal requirement for owners of dogs to pick up their dog's faeces on rural and agricultural land (Great Britain, 2011, 2014; Scottish Government, 2003). Hunting activities occur at a fast pace and with the pack often out of sight of hunt participants which makes the collection of faeces voided by the pack impossible. There are currently no guidelines or legislation in the UK relating to the fouling of hound packs on land used for hunting.

Hunt packs may attend public events outside of hunt meets, such as hound shows, agricultural shows and county fairs. Such events are likely to lead to increased contact between humans, especially children, and hounds. The risk of zoonotic disease transmission, such as infections with enteric bacteria and parasites, will be highest through petting, touching, feeding and being licked by the hounds (Harris & Dorning, 2017). The increased risk of transmission of zoonotic parasites such as

Toxocara canis and *E. granulosus* to children through such activities has been recognised (Theodoridis *et al.*, 2001; Eckert & Deplazes, 2004; Moro & Schantz, 2009).

As well as 'sporting visits' where hounds from one hunt in the UK will visit another, hunts will also travel to mainland Europe to hunt jointly with overseas packs (Cunningham & Cunningham, 2014). Equally, UK hunts will host packs from mainland Europe (Holliday, 2017). In such events, hunts have to meet current EU pet travel conditions, including administration of a praziquantel-based wormer 1-5 days prior to UK entry (DEFRA., 2019). Outside of the hunting season, many hunts can be disbanded and sent or 'billeted' to farms for kennelling (Boufana *et al.*, 2015).

Diseases causing morbidity and mortality in hunting hounds are likely to be underreported; hunts will typically cull sick or underperforming hounds without investigating potential health issues, may not seek veterinary treatment and favour culling to maintain the healthiest hounds (Harris & Dorning, 2017). It is estimated that hunts registered with the MFHA cull approximately 3000 hounds per year due to their inability to hunt with the rest of the pack (Burns *et al.*, 2000).

A range of infectious diseases have been reported in hunting hounds worldwide and reviewed by Harris and Dorning in their report of potential disease risks of hunting with hounds (Harris & Dorning, 2017). A detailed review of these falls outside the scope of this study, however in recent years, UK hunt packs have been under scrutiny for their potential role in the spread of diseases important to animal and public health. High-profile disease outbreaks involving UK hunting hounds, particularly with links to feeding of raw meat, have prompted investigations into the biosecurity risks associated with hunting activities.

In 2016, an outbreak of bovine tuberculosis (bTB) in a UK foxhound pack led to a coordinated epidemiological investigation by APHA, Public Health England (PHE) and the University of Edinburgh and resulted in the cull of 85 hounds testing positive for infection. Possible routes of infection included contact with bovine cases or being fed contaminated meat (Phipps *et al.*, 2018). In 2002, a retrospective investigation of a

virulent outbreak of respiratory disease in a pack of English foxhounds, in which one hound died and six were euthanised, identified the cause as equine influenza A virus (H3N8). Although the mechanism of infection was not confirmed, it was suggested that hounds may have inhaled aerosolised virus while eating raw infected equine lung material (Daly *et al.*, 2008). In 1999, a foxhound from a UK hunt regularly fed raw bovine carcases demonstrated protracted faecal shedding of oocysts of *Neospora caninum*, a major cause of bovine abortion, for over 4 months (McGarry *et al*., 2003).

3.1.3.2. Echinococcoses and other cestode infections in UK hunting hounds

3.1.3.2.1. E. granulosus

A number of studies have identified *E. granulosus* in hunting hounds in the UK, although studies have largely focused on foxhound packs in mid-Wales and bordering regions, where incidence of human cases have historically been highest. A study of 121 hounds (mostly foxhounds) in 21 Welsh hunt packs reported 28.8% of hounds to be infected with *E. granulosus* (Thompson & Smyth, 1974). A larger study of 553 foxhounds in 12 hunt packs in Dyfed, Wales employing arecoline purgation techniques reported *E. granulosus* in 162/581 (29%) of hounds in 8/12 (66%) packs (Williams, 1976). More recent surveys have used coproELISA and coproPCR techniques and have included a small number of packs outside of known hotspot areas of infection, although the focus has remained on foxhounds. Two recent studies employing coproELISA and coproPCR techniques to screen foxhound packs in England and Wales for *E. granulosus* and *E. equinus* reported 93/364 (25.6%) faecal samples from 5/8 (62.5%) hunts as coproantigen positive for *Echinococcus* spp. In addition, 10/364 (2.8%) hunts were positive on coproPCR of *E. granulosus* (Lett *et al.*, 2018), including a hunt in Northumberland. Sequencing of one coproPCR product was later confirmed as *E. granulosus* (Boufana *et al.*, 2015).

The island of Ireland is not considered endemic for *E. granulosus* (Deplazes *et al.*, 2017). To the authors knowledge, *E. granulosus* has not been reported in dogs in Ireland. Human cases of hydatid disease have been reported in Ireland, although they

were either not considered to be autochthonous (Butler *et al.*, 2003) or the lesion was characterised by gross examination only (Logan, 1971).

Importantly, an autochthonous case of human cystic echinococcosis in the UK has been reported in a foxhound hunt worker. A 41-year old man with a 21-year history of working as a kennelman and huntsman for 3 foxhound packs in southwest England was diagnosed with a hepatic hydatid cyst requiring extensive surgery and treatment. *E. granulosus* (G1) was confirmed via histopathology, coproPCR and sequencing. A high probability of occupational transmission through the extended period of work with foxhounds was reported (Craig *et al.*, 2012).

3.1.3.2.2. E. equinus

E. equinus is endemic to the UK and has been isolated from horses, dogs and captive mammals; a Burchell's zebra (*Equus burchellii*) and a red ruffed lemur (*Varecia rubra*) (Thompson & Smyth, 1975; Kumaratilake *et al.*, 1986; Thompson & Eckert, 1986; Boufana *et al.*, 2012; Boufana *et al.*, 2015). In Ireland, *E. equinus* has been reported in dogs and horses (Hatch, 1970; Kumaratilake *et al.*, 1986). Transmission in the UK is believed to be mainly sustained through a hunting hound-horse lifecycle via the feeding of infected equid fallen stock to hounds and the subsequent parasite egg contamination of land grazed by horses (Thompson & Smyth, 1975; Lett, 2013;). The parasite is not currently considered zoonotic, although a putative human case in Asia has been recently reported (Discontools: Echinococcosis, 2019).

Changes in feeding practices of UK hunting hounds after the end of the Second World War led to a widespread increase in *E. equinus* transmission through the domestic hunting hound-horse lifecycle. Economic pressures, labour shortages and a rise in the cost of fuel led to a shift away from traditional feeding of hounds with boiled horse flesh mixed with oat meal porridge towards the feeding raw horse meat and offal (Thompson & Smyth, 1975). Before the 1940's, *E. equinus* was reported sporadically and in localised areas (Southwell, 1927; Thompson & Smyth, 1974). In the early 1970's, peak prevalence rates of over 60% were reported in horses at slaughter (Dixon, Baker-Smith & Greatorex, 1973; Thompson & Smyth, 1975). A survey of 21

hunt packs at the time found 11 (52.4%) harboured worms morphologically identified as *E. equinus* (Thompson & Smyth, 1975). A later survey of cystic echinococcosis in 1141 horses and ponies identified hydatid lesions in 544 (48.6%) of carcases at post-mortem inspection (Cranley, 1982).

The introduction of the Hunting Act (2004) was expected to bring a decline in *E. equinus* infection in horses due to a reduction in hunting activities and the contamination of grazing land by hunting hounds (Thompson, 2008). However, changes to the number of active hunts and coverage of land through hunting activities since the Act have been relatively minor (Harris & Dorning, 2017).

The studies by Lett *et al.*, which screened foxhound packs in England and Wales for *E. granulosus* and *E. equinus* (also described in section 3.1.3.2.1) reported 5/364 (1.4%) samples from 2/8 (25%) hunts testing positive for *E. equinus* by coproPCR (Lett, 2013; Lett *et al.*, 2018). Subsequent sequencing analysis of the five positive coproPCR samples confirmed their identity as *E. equinus* (Boufana *et al.*, 2015), suggesting that foxhounds still play an important role of in the transmission of *E. equinus* in the UK.

3.1.3.2.3. Other cestodes

In addition to *E. granulosus* and *E. equinus*, hunting hounds have been identified as hosts of several other cestode species of animal and public health importance. Studies, largely conducted in Wales, report carriage of *T. hydatigena*, *T. multiceps*, *T. ovis*, *T. pisiformis*, *T. serialis*, and *D. caninum* (Williams, 1976; Stallbaumer, 1987; Jones & Walters, 1992). To date, there is no evidence to support the endemicity of *E. multilocularis* in UK or Ireland (Deplazes *et al.*, 2017). The parasite has been reported in hunting hounds elsewhere; in a study of 289 dogs in Slovenia, of which 85 were hunting hounds, coproELISA and coproPCR identified 2/85 (2.4%) positive for *E. multilocularis* (Antolová *et al.*, 2009). *E. multilocularis* is a notifiable disease in animals in the UK and Ireland (European Commission, 2011). Passive surveillance in fox carcases is reported annually to the European Commission by the Food and

Environmental Research Agency (FERA) in the UK and the Department of Agriculture, Environment and Rural Affairs (DAERA) in Ireland.

3.1.4. Worming of hunting hounds

The Code of Practice of the Welfare of Hounds in Hunt Kennels is a set of husbandry guidelines issued to all hunts registered with the CHA (The Council of Hunting Associations, 2015). The Code has no statutory powers, but offers recommendations on health and welfare of hounds within the framework of the Animal Welfare Act (2006).

The Code advises that each hunt should have a Hound Health Programme, based on a checklist of measures and agreed by the Hunt Master, the kennel huntsman and the provider of veterinary services to the hunt. As part of preventative health measures, regular worming of the pack for roundworms, tapeworms and hookworms at least twice a year, at the start and end of the hunting season, is recommended. Hunt staff are advised to consult with their veterinary surgeon on the selection of worming products available. Worming for *Dipylidium*, *Taenia* and *Echinococcus* spp. with a praziquantel wormer is advised, and the risk to hounds and farm livestock from such parasites is acknowledged. The Code does not mention the risk of human infection from zoonotic *Echinococcus* spp. In contrast, the Code does advise on the zoonotic risk of *Toxocara* roundworms and the importance of appropriate worming to prevent it. All worming treatments carried out by a veterinary surgeon or a kennel huntsman must be recorded on a 'Use of Veterinary Medicines' sheet approved by the CHA (The Council of Hunting Associations, 2015). Baily's Hunting Companion, a compendium of information on hunting with hounds, suggests that hounds on a predominantly raw flesh diet should be wormed several times a year, however advising that a different medication is used on each occasion to prevent immunity developing to any one product (Dangar, 1994). There is little evidence of anthelmintic resistance in intestinal and extra-intestinal worms of dogs (ESCCAP, 2017). The first case series of resistance to praziquantel in *Dipylidium caninum*, a zoonotic cestode of dogs and cats, in five pet dogs in the United States, was recently published (Chelladurai *et al.*, 2018).

Recommendations for worming of hunting hounds in the scientific literature relating to *Echinococcus spp.* often adopt a risk-based approach (Deplazes *et al.*, 2011). The common feeding of raw meat and offal makes hunting hounds a high-risk group for infection and transmission of echinococcosis to livestock and humans (ESCCAP, 2017; Harris & Dorning, 2017; Lett *et al.*, 2018). As a minimum, recommendations are to dose orally with a praziquantel-based wormer at least four times per year (Craig *et al.*, 2012; Deplazes *et al.*, 2011). Praziquantel is the only widely available licensed treatment for *Echinococcus* spp. infection in dogs in the UK (National Office of Animal Health, 2017). Despite a cestocidal efficacy of >99%, it has little residual activity and is not ovicidal (Craig *et al.*, 2017; European Food Safety Authority, 2015). As such, dogs and hounds that are fed raw meat or offal or are able to scavenge carcases of fallen stock are at risk of re-infection. The pre-patent period of *E. granulosus* is between 42 and 45 days (Craig *et al.*, 2017a). While worming with praziquantel four times per year can, as a minimum, reduce the burden of infection (Budke, 2002; Craig *et al.*, 2017), only 6-weekly worming with praziquantel would effectively prevent a patent infection and egg shedding (Craig *et al.*, 2017; ESCCAP, 2017). The recommended treatment regimen for confirmed *Echinococcus* carriage is a two-day dosing with praziquantel under veterinary supervision and the bathing of the coat to remove any viable eggs present (ESCCAP, 2017). A questionnaire survey of anthelmintic treatments in 16 foxhound packs in England and Wales reported 56% of hunts worming without a praziquantel-containing product despite 75% of hunts feeding raw offal from several species of fallen stock (Lett, 2013; Lett *et al.*, 2018).

3.1.5. Feeding practices in hunting packs

3.1.5.1. Diet of UK hunting hounds

The diet fed to active packs of hunting hounds needs to meet their nutritional demands and provide the necessary additional energy during the hunting season. The Code of Practice for the Welfare of Hounds in Hunting Kennels gives guidelines on the nutritional requirements of different types of hound at different life stages. The Code also recognises that with balanced proprietary foods, hounds do not need to be fed an exclusive flesh-based diet and it would be better for their health not to feed an all-flesh diet alone (The Council of Hunting Associations, 2015). However,

most hunts continue to feed raw meat and offal to their hounds, exclusively or otherwise (Harris & Dorning, 2017). In a study of 16 foxhound packs in England and Wales, 50% reported feeding raw offal from fallen stock, including cattle, calves, sheep, lamb and horses (Lett *et al.*, 2018). Hunts may include other foods in the hound diet, such as cereal-based porridges, fish and catering waste (Dangar, 1994; Lett *et al.*, 2018).

The feeding of raw meat and offal from livestock has been identified as a risk factor for definitive canine host infection with cestode parasites, including *E. granulosus*, *T. ovis* and *T. hydatigena*, and is a perpetuating factor in transmission of these parasites (Harris & Dorning, 2017; Otero-Abad & Torgerson, 2013; Torgerson, 2013). The Code recognises the potential *Echinococcus* infection risks associated with the feeding of raw meat products and suggests how to reduce them; it states:

"Masters and kennel huntsmen should be aware that raw flesh may be contaminated with infectious organisms such as salmonella and tapeworm cysts. The risk of transmission is considerably reduced if the evisceration and cutting up process is carried out hygienically so that flesh does not become contaminated by gut contents or the contents of tapeworm cysts. The risk is also considerably reduced if sheep carcases are not used, as sheep are the principal carriers of tapeworm cysts." (The Council of Hunting Associations, 2015).

3.1.5.2. Hunts as collectors and users of Animal By-Products (ABP) including fallen stock

Under EU Animal By-Products Regulation 1069/2009 (2009), Animal-By-Products (ABP) are classified as whole carcases, animal parts or materials not destined for human consumption. ABPs are divided into three categories defined by the risk they pose to public and animal health. Category 1 ABPs are classed as very high-risk and include whole or part carcases of animals known or suspected of being infected with a transmissible spongiform encephalopathy (TSE) (Article 8, Council Regulation 1069/2009) or containing Specified Risk Material (SRM) (Article 3(1)(g), Council Regulation (EC) 999/2001). Fallen stock of ruminant origin is classed as Category 1 material and is not suitable for animal consumption. Category 2 ABPs include other

high-risk materials such as fallen stock and animals or animal parts rejected from abattoirs as having infectious diseases (Article 9, Council Regulation (EC) 1069/2009). Category 3 ABPs are the lowest risk and include carcases or animal parts passed as fit for human consumption but removed for commercial or aesthetic reasons, not because they are unfit to eat (Article 10, Council Regulation 1069/2009).

Raw category 3 ABPs, collected from abattoirs or butchers, may be fed to pets at home in England, Wales and Northern Ireland, but not Scotland (DEFRA and APHA, 2018). Pet animals are not permitted to be fed other categories of ABP in any form. In the UK, hunt kennels (also zoos, maggot farms and registered kennels) have a derogation from the law permitting them to feed certain category 2 ABPs to carnivores. The derogation can be applied to hunt packs providing a) the material comes from animals not killed or that did not die as a result of disease communicable to animals or humans; b) feeding comprises meat and bone material only, excluding the following offal: liver, kidney, respiratory tract including lung and trachea, heart, spleen, pancreas, gastrointestinal tract (including stomachs), omentum, udder and reproductive organs; c) hounds are regularly treated for *Echinococcus* spp. in accordance with the recommendations of a veterinary professional and records of treatment are kept for at least two years and made available for inspection (DEFRA, 2011b).

Any establishment handling ABPs must be approved or registered with APHA. A site that collects and treats ABPs to be used as animal feed is considered a collection centre. A site that feeds category 2 and 3 ABPs to animals is considered a final user. Collection centres of ABP (including hunts if registered to do so) process carcases to separate category 1 material (for appropriate disposal) in order to utilise the remainder of the carcase as category 2. Hunt kennels that collect fallen stock, process it and use the flesh as feed are considered as both collection centres and final users. Most active hunts are registered as approved ABP establishments (APHA, 2018).

Hunting packs may also register as collection centres or final users with the National Fallen Stock Company (NFSCo), a community interest company, established to organise logistics between farmers and other livestock producers and collectors and

users of fallen stock, including rendering plants, maggot farms and hunting kennels (NFSCo, 2019a). Farmers are responsible for the safe and legal collection of fallen stock via their own arrangements with an approved establishment to handle ABPs or via the NFSCo. As part of animal collection/disposal services, hunts may also humanely destroy animals to be taken away as fallen stock. Carcases can then be used as feed for the hunt or the hunt simply used as a disposal service, providing rules relating to processing of Category 1 material and disposal of unused ABP are adhered to (DEFRA, 2014).

Abattoirs may consign category 2 ABP to hunt packs registered and approved as final users with APHA. Such ABP must be stained with a colouring agent and labelled as 'For feeding to (the species of animal intended)' (FSA, 2018b).

Unused or leftover ABPs at hunting kennels must be sent to an approved processing or incinerating plant or incinerated on-site if the hunt premises are also approved as both an ABP handling site and incineration site (DEFRA, 2014). Any cattle over 48 months of age collected as fallen stock must be tested for transmissible spongiform encephalopathies (TSEs) within 72 hours of death. In such cases, a hunt may provide a collection service but the carcase must still go to an approved site for TSE testing (DEFRA, 2014).

3.1.6. Study aims

Surveillance of echinococcosis in dogs is the most effective method of assessment of public health risk and evaluation of control programmes (Craig *et al.*, 2015; Craig & Larrieu, 2006). Evidence to date suggests that hunting packs play an important role in the transmission of *Echinococcus* spp. in the UK (Boufana *et al.*, 2015; Lett, 2013; Lett *et al.*, 2018; Thompson & Smyth, 1975; Williams, 1976). However, there is very little data on the risks posed by hunting packs operating outside the known prevalence hotspots of *E. granulosus* in Wales and by hunting packs other than foxhounds. A reported case of cystic echinococcosis in a UK hunt worker in the southwest of England (Craig *et al.*, 2012) highlights the need to build evidence at the

national level to better inform guidelines, practices and policies relating to hunting with hounds.

The study had three main aims: firstly, to investigate the husbandry and healthcare practices of different types of hunting packs active around the UK; secondly, to assess the proportion and location of UK hunts testing positive for *Echinococcus* spp. infection; thirdly to analyse the resultant data for associations between coprological test positivity and husbandry factors important to the transmission dynamics of *Echinococcus* spp. including diets fed, land access, worming practices and collection and disposal of faeces.

The findings of this study contribute to the overarching thesis aim: a better understanding of *Echinococcus* spp. distribution and risks in the UK to inform surveillance, public health information and future control efforts.

3.2. Methodology

This section describes study design and participant recruitment. Laboratory methods common to the three canine studies are summarized in brief here and described in detail in Chapter 2. Any modifications specific to this study design and recruitment strategy are described here.

3.2.1. Study design

This was a prospective, cross-sectional prevalence survey of *Echinococcus spp*. in hunting hounds in the UK. The source population was all hunts registered in Baily's Hunting Directory (Baily's Hunting Directory, 2018) in March 2016 and the study unit was the individual hunt pack. Participation was by mail and/or email invitation to the hunt correspondence address listed in the directory.

At the time of sampling, 367 hunts were listed in the UK in Baily's Hunting Directory (Baily's Hunting Directory, 2018). Inclusion criteria for participation were a) registration in the directory as hunts engaging in trail hunting, drag hunting or live quarry hunting as permitted by exemptions to the Hunting Act (Great Britain, 2004) and b) availability of a correspondence address and/or email for contact. A summary

of hunts by hunt type registered in the UK at the time of sampling is shown in Appendix I-a.

Currently, very little data are available on the prevalence of *Echinococcus* spp. in hunting packs outside areas of known prevalence in Wales and neighbouring English counties. To detect as hunt as infected based on pooled faecal sampling of individual hounds, a sample size based on a prevalence of 10% *Echinococcus* spp. infection at the hound level in the study population was estimated, based on limited existing data of coproPCR and coproELISA-based *Echinococcus* spp. prevalence studies. Two separate reports of coproPCR hound-level prevalence in Welsh and English hunt packs reported 16.7% (1/6 hounds, number of hunts unknown) (Boufana *et al.*, 2015) and 3.8% (14/364 hounds) (Lett, 2013). However, both reports used samples from the same group for their analyses. The latter study also reported a hunt-level coproPCR positivity of 87.5% (7/8 hunts). Lett also undertook coproELISA testing of the 8 participating hunts and returned 25.5% (93/364) hound and 62% (5/8) pack coproELISA positivity (Lett, 2013; Lett *et al.*, 2018). Both studies were undertaken in areas of known high incidence in mid-Wales and neighbouring English border regions. The use of an estimated pack prevalence based solely on data from high prevalence regions could result in a sampling frame unsuitable for detection in areas of anticipated low prevalence. An estimated hound-level prevalence of 10% offered a conservative estimate, given the wider scope of the current study into other parts of the UK thought to be non-endemic for the parasite. Calculating the miminum sample size (n_{inf}) to estimate prevalence of disease using the following formula (Dohoo, 2010):

$$n_{inf} = \frac{(P)(1-P)Z^2}{d^2}$$

with an estimated infection prevalence (*P*) of 10%, the stadard degree of confidence (Z) of 1.96 for 95%CI and the desired precision (d) of +/-0.05 (half the desired CI width), the estimated sample size would be 138 samples (assuming infinite population size) Adjusted for clustering at the hunt level using the formula (Dohoo, 2010):

$$n' = n_{inf}[1 + p(m - 1)]$$

with an estimated total target population size (based on 367 total registered hunts with average 50 dogs per hunt), an estimated intra-class correlation coefficient (*p*) of 0.4 (based on farm dog *Echinococcus* spp. prevalence studies in the UK) (Mastin *et al.*, 2011) and estimated sample size (*m*) of 15 randomly-selected faecal samples per hunt, a total adjusted sample size (*n'*) of 911 hounds from 61 kennels was calculated.

3.2.2. Participant recruitment

During the period of March 2016 to June 2016, the author focused on efforts to engage and recruit members of the hunting community to the study in different ways. Firstly, a letter of introduction, outlining the design, aims and objectives of the study was sent to the Hunting Office, the Secretary of each of the hunt associations in England, Wales and Scotland and the chairman of the Hunting Association of Ireland (for participation of hunts in Northern Ireland) (Appendix I-b). The letter was followed by a telephone call from the author to discuss the study and invite the Associations to offer their written support ahead of mail invitations to all registered hunts. The Hunting Office, on behalf of the hunting associations agreed to endorse the study and contact the secretaries of the larger hunt associations to discuss participation further.

Secondly, the author contacted Professor The Lord Alexander Trees, crossbench member of the House of Lords and professor of veterinary parasitology, to request his support for the study. This was received in the form of a quote highlighting the importance of the study and urging participation of hunts. Permission was granted by Lord Trees to use the quote in all study promotional material. Thirdly, the author contacted the editorial team of Baily's Hunting Directory to arrange for a summary of the forthcoming study to be included in the online news bulletin of the Directory website, timed to coincide with forthcoming mail invitations to the study. Fourthly, to further encourage a high response rate, an information email to the registered Hunt Master, Kennel Man or Secretary was sent one week before mail invitations, advising members to observe for arrival of the study pack and to take part. Finally,

the recruitment period coincided with the season of county agricultural shows in the UK, at which hunt associations often display hounds as part of a showcase of wider countryside pursuits. The author attended several agricultural shows in England and Wales to meet hunt staff and the staff of Baily's Hunting Directory to raise awareness of the study and encourage participation. The author elected not to attend any hunt meets during the recruitment process in order to maintain a non-partisan position as a researcher on the subject of hunting with hounds.

A participant information sheet and a study questionnaire (Appendix I-c, I-d) were designed using Microsoft Word 2010 (Microsoft Corporation, USA). In order to recruit as many hunts to the study as possible, questionnaire and faecal sampling kits were sent to all listed hunts in the register, addressed to the named Hunt Master at the correspondence address provided for the hunt. A recruitment period of 6 months was selected to allow sufficient time for hunts to take part and have any queries relating to the study addressed by the author. Particular emphasis was placed in all communications that participation was voluntary and anonymized, with no hounds, hunts or their staff identified in any study output. Questionnaires and sampling kits were identified by a unique code and the database of records was available only to the researcher and the project supervisory team. Hunts and hunt associations were advised that individual results of faecal testing would not be reported to participants, however general anonymized study findings would be made available in the form of a summary report to the hunt associations and any participating hunts requesting them. Response rate was monitored and when the rate began to decline, approximately one month after deployment of sampling kits, a reminder email was sent to non-participating hunts to encourage a response.

3.2.3. Questionnaire and faecal sampling

An anonymized survey questionnaire on the husbandry and worming practice of hunting hounds and faecal sampling kit (as described in Chapter 2) was sent by Royal Mail to each registered hunt with instructions for pre-paid return. Each hunt was asked to collect a total of 16 samples from different faecal pats divided equally into the two sample pots provided. This represented 2-3g from each of 16 faecal pats,

remaining within the permissible volume of faeces transportable as a biological sample by Royal Mail. Hunts were requested wherever possible to ensure that each sample came from a different hound in the pack.

3.2.4. CoproELISA for *Echinococcus spp*.

Pooled faeces samples received were frozen at -80˚C for minimum of 1 week prior to further processing to destroy infective stages of cestode parasites (Carabin *et al.*, 2005; Eckert *et al.*, 2002; van Kesteren, 2015) and stored at -20˚C thereafter.

CoproELISA was undertaken as described in detail in Chapter 2. In brief, all samples were analysed for *Echinococcus spp.* coproantigen using a genus-specific polyclonal sandwich coproELISA protocol as described by Allan *et al.* (1991) and Craig *et al.* (1995) further optimised at the University of Salford by van Kesteren *et al.* (2013). A cut-off value of 0.1221 OD for positive/negative test outcome was used, based on the mean OD value plus 3 standard deviations for a panel of negative control samples (canine faeces from a wormed cohort of dogs in the Falkland Islands) as described in Chapter 2.

Further negative control samples were from pet dogs in England volunteered by colleagues of the researcher; dogs were fed solely non-raw proprietary dog food and were recently wormed with praziquantel. Positive control samples were from known *E. granulosus* G1 infected dogs in Kyrgyzstan confirmed by coproELISA or purgation in previous studies (van Kesteren *et al.*, 2013, M.Rogan, personal communication).

3.2.5. CoproPCR for *Echinococcus spp.*, *E. granulosus* (G1) and *E. equinus* (G4)

Chapter 2 describes in detail the coproPCR protocols used in this study. CoproDNA was extracted from faecal samples using a QIAGEN QIAmp® DNA Stool kit following manufacturer's instructions for larger volume faecal samples (QIAGEN, 2014). The presence of genus-specific *Taenia* spp. and *Echinococcus* spp. coproDNA was investigated using the multiplex protocol described by Trachsel *et al.* (2007). The protocol by Abbasi *et al.* (2003) was used to identify *E. granulosus* sensu lato (s.l). Species-specific *E. granulosus* G1 and *E. equinus* coproDNA were investigated using

protocols by Boufana *et al.* (2013) and Lett *et al.* (2018) respectively. PCR amplification products were identified from their size using gel electrophoresis and products were sequenced for further confirmation (Source Bioscience, UK).

3.2.6. Statistical analysis

Analysis and graphical display of coproELISA data was performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA). Descriptive and inferential statistical analyses were performed using Stata 14 (StataCorp, 2015). Continuous questionnaire variables were tested for normality using the Shapiro-Wilk or D'Agostino-Pearson tests and visualized in a Normal Q-Q plot. To describe proportions of hunts engaging in the different husbandry and healthcare practices studied questionnaire data are presented as proportion, percentage and 95% Confidence Interval (95%CI) of the percentage. A two-sided Fisher's exact test was used to investigate associations between coprological test positivity and *a-priori* risk factors for infection at the hunt level, including hunt type, geographical location, raw food feeding and sub-optimal worming, with significance set at $p<0.05$.

3.3. Results

3.3.1. Study response and participation

At the time of sampling, 367 hunts were registered in Baily's Directory, with 6 listed as 'disbanded' and 313 supplying complete correspondence details. During August-September 2016, questionnaires and sampling kits were sent to the 313 hunts providing a mailing address, with an accompanying introduction email if details were provided. The study received responses from 37/313 hunts, giving a response rate of 11.8% (95%CI: 8.2-15.4). Five responses were to inform that the hunt was no longer active, the person contacted was no longer hunt staff or the hunt had amalgamated with another. The study received 32/313 completed questionnaires and 30 faecal samples during the 6-month recruitment period, giving an overall participation rate of 10.2% (95%CI: 6.9-13.6). Providing hunts adhered to collecting a sample from each of 16 faecal pats, aiming to represent 16 different hounds, this would represent samples from 480 hounds in total.

3.3.2. Hunt distribution and participation

Foxhound packs were notably the only group registered in every devolved country in the UK, with packs in England the largest overall hunt group, representing 45% (142/313) of all listed UK hunts. Many pack types were not registered in any country or had very low numbers listed. The highest study participation rates were in the Basset 20% (2/10) and Harrier 17.3% (4/23) groups, although their overall hunt numbers were low. No drag hound, mink hound, rabbit hound or stag hound packs responded to the study and their hunt types will not be considered further in the results.

Table 3-1. Number of hunts listed per country and hunt type at the time of study sampling, including number participating and participation rate.

Hunt type		England	Wales	Scotland	Northern Ireland	Total
Foxhounds	Total	142	27	9	6	**184**
	Study	12	3	3	0	**18**
	%	8.5	11.1	33.3	0	**9.8**
	(95%CI)	(3.9-13.0)	(0.0-23.0)	(2.5-64.1)	(0.0)	**(5.5-14.1)**
Beagles	Total	50	4	0	3	**57**
	Study	5	2	0	0	**7**
	%	10.1	50	0	0	**12.3**
	(95%CI)	(1.7-18.3)	(1.0-99.0)	(0.0)	(0.0)	**(3.8-20.8)**
Bloodhounds	Total	11	1	0	1	**13**
	Study	1	0	0	0	**1**
	%	9.1	0	0	0	**7.7**
	(95%CI)	(0.0-26.1)	(0.0)	(0.0)	(0.0)	**(0.0-22.2)**
Harriers	Total	17	0	0	6	**23**
	Study	2	0	0	2	**4**
	%	11.8	0	0	33.3 (0.0-71.1)	**17.4**
	(95%CI)	(0.0-28.1)	(0.0)	(0.0)		**(1.9-32.9)**
Bassets	Total	8	1	1	0	**10**
	Study	1	0	1	0	**2**
	%	12.5	0	100	0	**20.0**
	(95%CI)	(0.0-35.4)	(0.0)	(NA)	(0.0)	**(0.0-24.8)**
Minkhounds	Total	10	2	1	0	**13**
Draghounds	Total	7	2	0	0	**9**
Staghounds	Total	3	0	0	0	**3**
Rabbit dogs	Total	1	0	0	0	**1**
Total hunts	**Total**	**249**	**37**	**11**	**16**	**313**
	Study	**21**	**5**	**4**	**2**	**32**
	%	**8.4**	**13.5**	**36.4**	**12.5**	**10.2**
	(95%CI)	**(5.0-11.9)**	**(2.5-24.5)**	**(7.9-64.8)**	**(0.0-28.7)**	**(6.9-13.6)**

The distribution of registered hunts by country varied widely, with the majority, 79.5% (249/313) listed in England, followed by 11.8% (37/313) in Wales, 5.1% (16/313) in NI and 3.5% (11/313) in Scotland. Scotland showed the highest overall study participation rate of 36.3% (4/11), followed by 12.5% (2/16) in NI, 10.8% (5/37) in Wales and the lowest rate of 8.8% (22/249) in England. A summary of hunts registered in England, Wales, Scotland and Northern Ireland by hunt type, together with study participation is shown in Table 3-1.

3.3.3. Structure of participating hunts

The mean number of hounds (all types) per hunt was 67 (SEM: 5, 95%CI: 57-77), with the mean number of males (≥6 months) 27 (SEM: 2, 95%CI: 23-31), females (≥6 months) 35 (SEM: 3, 95%CI: 29-41) and pups (<6 months) 4 (SEM: 1, 95%CI: 3-7). Fig 3-1 shows the distribution of mean pack size according to male, female and pup groups in each of the participating hunt types.

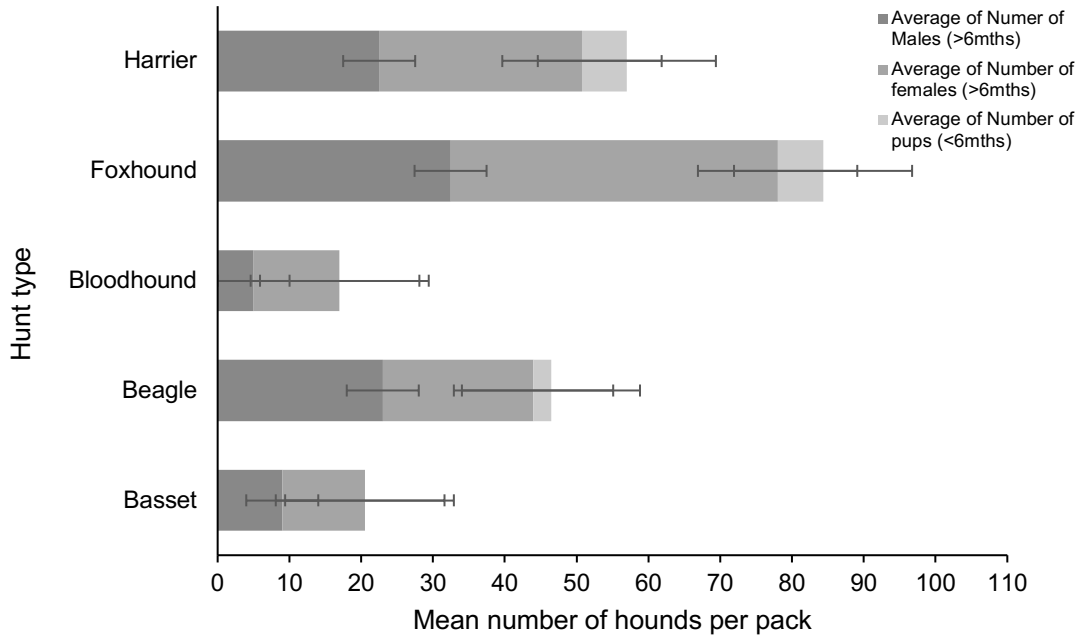

Figure 3-1 Mean number of hounds per pack type and by male (>6 months), female (> 6 months) and pup (<6 months) subgroup in a survey of 32 hunting packs in the UK. Error bars represent the SEM for each subgroup.

A total of 8/32 (23%, 95%CI: 10-40) hunts (all foxhound packs) also kept terriers as part of the hunt. Occasionally, hunts will house hounds from other packs at their

kennels. At the time of sampling, 8/32 (25%, 95%CI: 10-40) packs had housed hounds from another pack at their kennel within the last 12 months.

3.3.4. CoproELISA results

A hunt was classified as *Echinococcus* spp. coproELISA positive if one or both pooled hound faecal samples submitted gave a mean coproELISA OD reading above the defined cut-off value of 620nm 0.1221 OD.

In total, 5/64 (8.1%, 95%CI: 1.3-14.8) pooled samples tested positive on coproELISA (Fig. 3.2.). In two cases, both positive pooled samples came from the same hunt. Overall, the samples represented 3/32 (9.4%, 95%CI: 0.0-19.5) participating hunts.

Of 3 hunts testing positive on coproELISA, one hunt was located in the South West of England, one hunt in the North West of England and one hunt in the Scottish Borders.

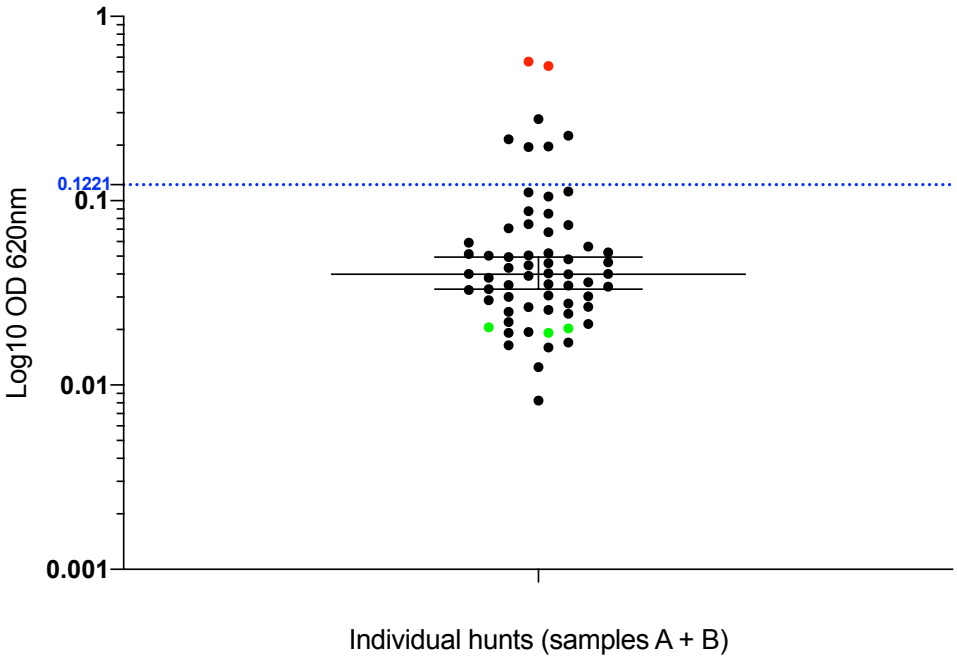

Figure 3-2. Echinococcus spp. CoproELISA results for pooled hound pack faecal samples from 32 UK hunts participating in the survey *OD 620nm readings are shown as a log scale with ● = positive control and ● = negative control. The cut-off value for test positivity is shown as 0.1221 OD 620nm. Horizontal lines represent the mean OD and 95%CI.*

No statistical significance was found between country of origin of the hunt and coproELISA positivity (p=0.732).

Table 3-2. Summary of genus-specific Echinococcus coproELISA positive samples and controls from a cross-sectional study of 32 hunts in the UK

Hunt ID /control	Mean OD	Region	Country
44A*	0.2772	South West	England
44B*	0.2154	South West	England
47A*	0.1958	North	England
47B*	0.2251	North	England
81B	0.1970	Borders	Scotland
Pos 1	0.5686	na	Kyrgyzstan
Pos 2	0.5383	na	Kyrgyzstan
Neg 1	0.0206	na	England
Neg 2	0.0203	na	England
Neg 3	0.0192	na	England

*Individual hunts are identified by a numerical code and pooled samples identified as A and B * denotes both samples submitted from the same hunt. [a]Negative faecal sample 'spiked' with E. granulosus whole worm extract to give a positive control sample. Cut-off value for a positive result was OD 620nm 0.1221. Pos = positive control, Neg = negative control, na= not applicable. All samples were assayed in triplicate and a mean OD value is given.*

All 3 hunts testing positive on coproELISA were foxhound packs, however there was no overall statistically significant association between hunt type and a coproELISA positive response (p=0.784). A summary of coproELISA results for positive samples and controls is shown in Table 3-2.

3.3.5. CoproPCR results

A single pooled faecal sample from 1 hunt (Hunt 23) out of 32 (3.1%, 95%CI 0.0-9.2) tested positive on coproPCR for *E. granulosus* G1 by amplification and visualisation of a 226bp DNA fragment within the NAHD dehydrogenase subunit (ND1) of the mitochondrial gene. The hunt was located in the northwest of England. Unfortunately, it was not possible to successfully isolate a viable DNA product from positive samples for sequencing as a confirmatory step. Neither the genus-specific multiplex coproPCR to identify *Taenia* spp. and *Echinococcus* spp. or the *E. granulosus* s.l. coproPCR identified any positive samples despite troubleshooting to improve test specificity and sensitivity.

3.3.6. Comparison of coproELISA and coproPCR results

Pooled faecal samples from a total of 3 hunts tested positive for *Echinococcus* spp. coproantigen and one hunt tested positive for *E. granulosus* G1 coproDNA. Although it was not possible to undertake a statistical comparison of agreement between the tests using Cohen's Kappa statistic, the sole coproDNA positive sample found did not test positive for coproantigen, nor was coproDNA identified in any samples that had tested positive on coproELISA. This would suggest little agreement between the coprodiagnostic tests in this study. A summary table of associations between coprodiagnostic test outcomes and questionnaire variables is shown in Appendix I-e.

3.3.7. Hound diet

The questionnaire section on feeding regimens gathered data about frequency of feeding, type and source of food for the hounds (Appendix I-f). The majority of hunts, 30/32 (93.75%, 95%CI: 85.4-100), fed their hounds a single meal per 24-hour period, with 2/32 (6.25%, 95%CI: 0.0-14.6) feeding two meals in 24 hours.

Feed categories included raw flesh or viscera from fallen stock, raw flesh or viscera from an abattoir or butchers (i.e. having undergone meat hygiene inspection), cooked meat or viscera from any source, proprietary commercial dog food, catering waste, fish or other (open question option). Categories involving flesh or viscera were also classified into the species of origin, including sheep, lamb, beef, calf, pig, horse, donkey, goat and poultry. Overall, 18/32 (56.3%, 95%CI: 39.1-73.4) hunts reported being registered as collection centres for fallen stock within the National Fallen Stock Company (NFSCo) scheme.

Of hunts sampled, 27/32 (84.4%, 95%CI: 71.8-97.0) fed more than one type of diet to their hounds. The most common food type fed to hounds was raw meat from fallen stock of cattle or sheep origin, fed by 23/32 (71.9%, 95%CI: 56.3-87.5) hunts. Fewer hunts fed viscera from the same source, with 14/32 (43.8%, 95%CI: 26.6-60.9) doing so. Feeding of commercial proprietary dog food was common among the hunts, with 22/32 (68.8%, 95%CI: 52.7-84.8) doing so, although only 5/32 (15.6%, 95%CI: 3.0-28.2) were doing so exclusively. Hunts feeding exclusively commercial dog

food were the only group feeding a single diet source. Only one (foxhound) pack was cooking high risk and equid ABP prior to feeding. A summary of diet types fed is shown in Table 3-3.

Table 3-3. Summary of types of diet fed to hunting hounds in a cross-sectional survey of 32 hunting packs in the UK. Hunts recorded may be feeding more than one diet type. Data are shown as number and percentage of hunts feeding a food type with 95% Confidence Interval (95%CI)

Diet type		Number of hunts	Percent of total hunts*	95% CI
Fallen stock (Meat)	Cattle/Sheep	23	71.9	56.3-87.5
	Equid	19	59.4	56.3-87.5
	Other mammals	19	59.4	42.4-76.4
	Poultry	3	9.4	0.0-19.5
Fallen Stock (Viscera)	Cattle/Sheep	14	43.8	26.6-60.9
	Other mammals	10	31.3	15.2-47.3
	Equid	10	31.3	15.2-47.3
	Poultry	1	3.1	0.0-9.2
Abattoir/butcher (Meat)	Cattle/Sheep	7	21.9	7.6-36.2
	Equid	1	3.1	0.0-9.2
	Other mammals	6	18.8	5.2-32.3
	Poultry	1	3.1	0.0-9.2
Abattoir/butcher (Viscera)	Cattle/Sheep	3	9.4	0.0-19.5
	Equid	0	0	NA
	Other mammals	1	3.1	0.0-9.2
	Poultry	0	0	NA
Cooked (Meat)	Cattle/Sheep	1	3.1	0.0-19.5
	Equid	0	0	NA
	Other mammals	0	0	NA
	Poultry	0	0	NA
Cooked (Viscera)	Cattle/Sheep	1	3.1	0.0-19.5
	Equid	0	0	NA
	Other mammals	0	0	NA
	Poultry	0	0	NA
Other	Commercial	22	68.8	52.7-84.8
	Catering waste	1	3.1	0.0-19.5
	Fish	3	9.4	0.0-19.5
	Other	2	6.3	0.0-14.6

*Hunts were feeding more than one type of food

For analysis relating to potential risk of UK endemic *Echinococcus* spp. transmission to the canine host, feed types were further grouped into *E. granulosus* very high-risk (raw meat/viscera from any sheep or cattle fallen stock), *E. granulosus* high-risk (any source of raw flesh or viscera from any putative *Echinococcus* spp. host) and specific risk of *E. equinus* infection (any source of raw flesh or viscera from horse or donkey). A further low-risk category for *Echinococcus* spp. infection included the feeding of only commercial, cooked or catering waste food.

Very high-risk feed types were fed by 23/32 (71.9%, 95%CI: 56.3-87.5) hunts; high-risk feeds were fed by 25/32 (78.1%, 95%CI: 63.8-92.4) hunts; feed types with risk of *E. equinus* transmission were fed by 19/32 (59.4%, 95%CI: 42.4-76.4) hunts and low-risk diets only were fed by 5/32 (15.6%, 95%CI: 3.0-28.2) hunts.

Although there was no statistically significant association between risk category of diet fed and a positive result on coproELISA or coproPCR, there was a general trend, with all hunts testing positive on either coprodiagnostic test feeding all three categories of very-high risk, high-risk and *E. equinus* risk diets. No hunts testing positive were feeding an exclusively low-risk diet (Appendix I-f).

There were statistically significant associations between hunt type and the feeding of a very high-risk diet ($p<0.001$), a high-risk diet ($p<0.005$) and a low risk diet only ($p<0.01$). More specifically, foxhound packs were significantly associated with feeding very high-risk diets ($p<0.001$), high-risk diets ($p<0.01$), *E. equinus* risk ($p<0.001$) and exclusively a low-risk diet ($p<0.05$). The direction of association of the latter relating to hunts not feeding exclusively a low-risk diet. Beagle packs were significantly associated with feeding of a high-risk diet ($p<0.05$). A summary of associations between hunt type and diet risk category is shown in Table 3-4.

Table 3-4. Summary of Echinococcus spp. transmission risk by categories of feed to different types of hunting hound packs. Data are shown by total number of hunts participating in the study, the number and % of hunts feeding a risk category of food and 95% Confidence Intervals (95%CI).

Hunt type		*E. granulosus* very high risk	*Echinococcus* spp. high risk	*Echinococcus* spp. Low risk	*E. equinus* high risk
Foxhounds	Total hunts	18	18	18	18
	Total feeding	18	18	0	16
	%	100	100	NA	88.9
	(95%CI)				(74.4-100.0)
	P-value	<0.001	<0.001	<0.010	<0.001
Beagles	Total hunts	7	7	7	7
	Total feeding	3	3	3	2
	%	42.9	42.9	42.9	28.6
	(95%CI)	(6.2-79.5)	(6.2-79.5)	(6.2-79.5)	(0.0-62.0)
	P-value	=0.076	<0.05	=0.057	=0.091
Bloodhounds	Total hunts	1	1	1	1
	Total feeding	0	0	1	0
	% (95%CI)	0	0	100	0
	P-value	0.281	0.219	0.188	0.406
Harriers	Total hunts	4	4	4	4
	Total feeding	2	3	1	1
	%	50.0	75.0	25.0	25.0
	(95%CI)	(1.0-99.0)	(32.6-100.0)	(0.0-67.4)	(0.0-67.4)
	P-value	0.577	1.000	0.512	0.279
Bassets	Total hunts	2	2	2	2
	Total feeding	0	1	1	0
	%	0	50.0	50.0	0
	(95%CI)		(0.0-100.0)	(0.0-100.0)	
	P-value	0.073	0.395	0.292	0.157
Total hunts	Total hunts	32	32	32	32
	Total feeding	23	25	6	19
	%	71.9	78.1	18.8	59.4
	(95%CI)	(56.3-87.5)	(63.8-92.4)	(5.2-32.3)	(42.4-76.4)
	P-value	0.000	0.001	0.004	0.000

3.3.8. Storage and disposal of food

Respondents feeding any raw food items to the hounds were asked about the storage methods of these foods. Storing raw food at room temperature was the most common method used, with 22/27 (81.5%, 95%CI: 66.8-96.1) hunts doing so.

Comparatively few hunts reported other storage methods, including 3/27 (11.1%, 95%CI: 0.0-23.0) hunts storing raw food in a freezer, 6/27 (22.2%, 95%CI: 6.5-37.9) storing food in a refrigerator, 4/27 (14.8%, 95%CI: 1.4-28.2) drying food by fan before storage at room temperature and 1/27 (3.7%, 95%CI: 0.0-10.8) storing food in a cool box.

Participants were asked to give information about the methods used for the disposal of any unused ABP material; 23 out of 27 hunts responded to the question. Most hunts, 19/23 (82.6%, 95%CI: 67.1-98.1), disposed of ABP material via a rendering plant; 3/23 (13.0%, 95%CI: 0.0-26.8) disposed of material at an off-site incinerator and 4/23 (17.4%, 95%CI: 1.9-32.9) indicated that they returned ABP material to the original supplier.

3.3.9. Worming of hounds

Hunts were asked to provide details on worming product(s) used, dosage and frequency of administration, date of last worming and personnel responsible for worming the hounds. Where known, the active ingredients of the product and manufacturer guidelines for dosing were included.

All 32 participating hunts reported regularly worming their hound pack. A total of 15 different worming products (by trade name) were used by participating hunts, of which 9 were licensed for anthelmintic use in dogs in the UK (NOAH, 2017). All licensed products in use were for oral administration and were combination products for tapeworm and roundworm treatment or sole roundworm treatment. Licensed products included two available by veterinary prescription only (POM-V), five products supplied by a vet, pharmacist or trained specialist without prescription (NFA-VPS) and two products available as general sale (AVM-GSL). Several tapeworm treatments were included in the licensed combination products, including Praziquantel, Nitroscanate, Pyrantel and Piperazine Citrate. Six licensed treatments contained praziquantel as an active ingredient.

Licensed products were used by 27/32 (84.4%, 95%CI: 71.8-97.0) hunts. Taking worming 4 times per year as a baseline minimum (or following product instructions

issued to that effect) as advised by ESCCAP for non-risk assessed dogs (ESCCAP, 2017), 8/27 (29.6%, 95%CI: 12.4-46.9) hunts used their products according to this minimum. Wormers containing praziquantel as an active ingredient were used by 18/27 (66.7%, 95%CI: 48.9-84.4) hunts using a licensed product, although of these, only 5/18 (27.8%, 95%CI: 7.1-48.5) hunts were using them according to manufacturer's instructions or the baseline minimum protocol above. Two out of four packs testing positive for *Echinococcus* spp. on coproELISA or coproPCR were administering a wormer containing praziquantel, although neither were doing so by the protocol above.

Six worming products not licensed for used in dogs were being administered to hounds in 5/32 (15.6%, 95%CI: 3.0-28.2) hunts. These wormers included oral pastes for horses containing fenbendazole and injectable, oral drench and pour-on preparations for worming of livestock containing ivermectin or moxidectin.

An association between hunt type and use of a wormer containing praziquantel was close to statistical significance (p=0.055). Of all the hunt types, harrier packs were significantly more likely to administer a worming product that did not contain praziquantel (P<0.05).

Wales was the only country where all participating hunts administered a wormer containing praziquantel (p=0.052). Although not statistically significant (p=0.183), Northern Ireland was the only country where no participating hunts were administering a wormer containing praziquantel.

In 31 hunts responding with information on who administers wormers at the hunt kennels, 24/31 (77.4%, 95%CI: 62.7-92.1%) had wormers administered by a huntsman, 5/31 (16.1%, 95%CI: 3.2-29.1%) by a kennelman and 2/31 (6.5%, 95%CI: 0.0-15.1%) by the hunt master. No hunts indicated that a vet administered wormer to their hounds.

Almost all hunts, 31/32 (96.9%, 95%CI: 90.8-100.0%), reported having access to the Code of Practice for the Welfare of Hounds in Hunt Kennels document issued by the CHA to all registered hunts (The Council of Hunting Associations, 2015).

3.3.10. Land access and disposal of faeces

Table 3-5. Summary of land access to hounds in a cross-sectional survey of 32 UK hunt packs.

Site of access	Hunts	Percent	95%CI
Fenced grass yard for hounds only	22	68.8	52.7-84.8
Fenced concrete yard for hounds only	16	50.0	32.7-67.3
Open land with public access	22	68.8	52.7-84.3
Fenced field/area shared with livestock	20	62.5	45.7-79.3
Roads	8	25.0	10.0-40.0

Respondents were asked to describe the environment given to the hounds to exercise on a day-to-day basis, when not involved in seasonal hunting activity. It is accepted that the hounds will cross large distances of public and private rural land during hunting days. Results show hounds were exercised in a variety of environments, including land shared with livestock and with public access. Almost all hound packs, 31/32 (96.9%, 95%CI: 90.8-100), were exercised on land with public and/or livestock access. Further detail on hound access to different types of land is shown in Table 3-5.

Table 3-6. Summary of hound faeces disposal means by respondents in a cross-sectional study of 32 hunting packs in the UK.

Faeces disposal method	Hunts	Percent	95%CI
Rendering plant	3	9.4	0.0-19.5
Muck heap	12	37.5	20.7-54.3
With SRM[a]	3	9.4	0.0-19.5
Offal/flesh skip	3	9.4	0.0-19.5
Burning on site	2	6.3	0.0-14.6
Farmer	2	6.3	0.0-14.6
Waste disposal company	3	9.4	0.0-19.5
Slurry pit	1	3.1	0.0-9.2
Council refuse collection	2	6.3	0.0-14.6
No response	1	3.1	0.0-9.2

[a]Specified Risk Material

All participating kennels reported routinely collecting and disposing of hound faeces at the hunt kennels. Respondents were asked to describe the means by which any collected hound faeces were disposed. The most common means of faeces disposal was onto a muckheap, used by 12/32 (37.5%, 95%CI: 20.7-54.3) hunts. Further detail on faeces disposal method is shown in Table 3-6. Half of participating hunts, 16/32 (50%, 95%CI: 32.7-67.3), were disposing of faeces by means that could result in the contamination of agricultural or horticultural land if used as compost or fertilizer.

3.4. Discussion

3.4.1. Study participation

This study recruited 32 hunts, representing faeces collection from an estimated 480 hounds, assuming the sample collection instructions were followed. This fell below the 63 kennels and 945 hounds needed for inferential analysis of the data; therefore, the analysis reflects the findings within the sample group only. Nevertheless, the response represents the largest participation in a study of *Echinococcus* spp. in UK hunts using coproELISA and coproPCR methods to date, reflecting the willingness of some members of the hunting community to engage in research relating to the health of their hounds. In 2018, the MFHA held the first symposium on hound health and disease, to which hunt staff, farmers, researchers (including the author), civil servants and veterinarians working with hunts were invited. Echinococcosis and the associated disease risks of hunting with hounds were discussed at the meeting.

A total of 8/32 (23%, 95%CI: 10-40) hunts (all foxhound packs) also kept terriers as part of the hunt. The study did not differentiate between hounds and terriers in the sample collection or diet fed and worming regimes used for terriers did not differ from those of the hound pack.

Participant recruitment was a challenging aspect of the study for several reasons. Firstly, hunting with hounds remains a contentious activity that strongly divides public opinion. Concerns about perceptions of disease status in hunt packs amid considerable public and legal scrutiny might have limited the willingness of hunts to participate. These concerns could result in possible selection and information bias

from non-responses to survey questionnaires, possibly underrepresenting *Echinococcus* spp. and the associated transmission risks in this population. Secondly, contact details for hunts were obtained some months before an updated version of Baily's Hunting Directory was released. The previous update of the Directory had not been published for several years, so it was likely that many participation requests were sent to outdated addresses (Baily's Hunting Directory secretary, pers. comms). This was supported by the receipt of several letters and returned kits expressing this. Thirdly, at the study design stage, it was decided not to report individual test result to participating hunts, rather return overarching anonymised findings to the national hunting associations of the participating devolved nations. At the time, this decision was taken to avoid potential conflict between hunts who were sharing kennels on 'sporting visits' when relating disease findings. Not reporting results from the study to hunts may have discouraged some from taking part. In hindsight, the individual findings to each hunt could have been reported confidentially to a hunt member agreed in advance.

The inclusion of a randomisation step and individual sample collection could further address bias and allow prevalence estimation within different strata and within the desired confidence intervals.

3.4.2. CoproELISA and coproPCR results

The study identified three foxhound packs testing positive for *Echinococcus* spp. coproantigen; one hunt was located in the South West of England, one hunt in the North of England and one hunt in the Scottish Borders. To the author's knowledge, this represents the first reported positive *Echinococcus* spp. coproELISA test results for hunting packs in these three regions of the UK. No statistical significance was found between country of origin of the hunt and coproELISA positivity. While it was not possible to confirm these results via coproPCR, the finding supports evidence of *Echinococcus* parasite transmission in these regions. The only other study reporting *Echinococcus* spp. distribution based on coproELISA and coproPCR results reported *Echinococcus* spp. infection in two hunts in Northumberland (Lett *et al.*, 2018), bordering the Scottish Border region where most hunts in Scotland are located, and

within the North of England where this study identified hunts testing positive for *Echinococcus* spp. coproantigen. A foxhound pack in the South West of England tested positive for *Echinococcus* spp. coproantigen. To the author's knowledge, this is the first hunt in this region to do so. Although relating to probable infection several decades ago, the only UK report of occupational zoonotic transmission of *E. granulosus* relating to working with hunting hounds relates to packs located in the South West of England (Craig *et al.*, 2012).

This study identified a foxhound pack in the North West of England positive for *E. granulosus* (G1) on coproPCR. The study by Lett *et al.* (2018) did not identify *Echinococcus* spp. in a hunt tested within this region.

Contrary to the understanding that the highest UK prevalence of *E. granulosus* is in Wales, this study found no evidence of *Echinococcus* spp. infection in the 5 participating Welsh hunts. This is in contrast with existing research on echinococcosis in hunting hounds in Wales (Lett *et al.*, 2018; Stallbaumer, 1987). The reason for this is unclear, although it is possible that awareness of hydatid disease among the rural population in Wales, given previous education programmes may make it more likely that hunts will be aware of the importance of worming hounds. Indeed, in contrast to other parts of the country, all hunts from Wales in this study were using a praziquantel wormer.

E. equinus appears to occur sympatrically with *E. granulosus* in parts of Wales where the latter is known to occur (Boufana *et al.*, 2015). Despite the widespread feeding of ABP of equine origin reported in this study, no hunts tested positive for *E. equinus* on coproPCR. However, it is possible that the positive *Echinococcus* spp. coproantigen result in three hunt packs could be *E. equinus* or *E. granulosus*, although unfortunately, this was not confirmed by coproPCR.

While the assays used in this study are the mainstay for *in vivo* diagnosis of *Echinococcus* spp. in definitive canine hosts, there are limitations common to this study and the other canid studies in the thesis. The intermittent shedding of eggs in faeces, expected low worm burdens and the overdispersed nature of *Echinococcus*

spp. in dogs could lead to an underestimation of infection rates in individual and groups of animals (Torgerson & Deplazes, 2009). *Echinococcus* worm burden and population size can affect the sensitivity and specificity of coproELISA and coproPCR methods (Craig *et al.*, 2015; Deplazes *et al.*, 1992; Hartnack *et al.*, 2013). As with the other canid studies, PCR inhibitors in faeces, such as plant polysaccharides and bile salts, could interfere with the isolation of representative parasite DNA and PCR (Gunn & Pitt, 2012).

This study found a lack of agreement between coproELISA and coproPCR results, as was similarly encountered in the other HyData studies. Incongruent results were also encountered in a comparable coprological study of UK hunting packs, which reported discrepancies between individual hound test results in 3/8 hunts tested (Lett *et al.*, 2018).

This study identified 5 hunts that had administered a praziquantel containing wormer within 6 weeks of study participation. Praziquantel has a half-life of 3 hours in the intestine of the dog (EFSA Panel on Animal Health and Welfare, 2015), therefore retains little residual activity, allowing potential re-infection between the last worming dose and study sampling. CoproPCR is reliant on detection of DNA from voided *Echinococcus* spp. eggs (Craig *et al.*, 2015; Eckert *et al.*, 2002), infection may not be detected within the pre-patent period, however, coproELISA could detect infection. Coproantigen detection in faeces by coproELISA can occur within 5-10 days post-infection and does not depend on the presence of eggs in faeces (Eckert *et al.*, 2002). One hunt administering praziquantel reported worming with within 5 days of faecal sampling. A negative faecal antigen result with coproELISA has been reported 5 days following dosing with praziquantel (Deplazes *et al.*, 1992), so it is possible that this could affect the outcome of the test in this case.

Many samples submitted to the study contained relatively small volumes of faeces, suggesting that the desired number and amount of sample material were not collected in each case. The remote sampling strategy made maintaining consistency in sampling difficult. Coprophagia within the hound pack cannot be ruled out and may have affected the results relating to individual hounds. This has less impact here,

where the study unit is the hunt pack and not the individual hound. However, hounds ingesting faeces of other animals, including foxes or domestic dogs, when hunting or exercising, cannot be ruled out.

3.4.3. Hound diet

The most common food type fed to hounds was raw meat from cattle or sheep fallen stock, fed by 71.9% of hunts. Although hydatid cysts are more likely to occur in the liver and lungs of intermediate hosts, they can occur in muscle and other internal organs (Eckert *et al.*, 2002), therefore this feeding practice remains an infection risk. The feeding of raw meat to hounds presents a particular transmission risk of several other cestode parasites important to animal health, in particular *T. ovis* and *T. hydatigena* in sheep and *T. saginata* in cattle. The intermediate (larval) stages of these parasites cause considerable losses to the UK livestock industry through the condemnation of infected carcases (AHDB Better Returns Programme., 2018; SHAWG, 2019).

The feeding of raw offal from sheep and cattle was a common practice among participating hunts, with 43.8% of hunts doing so. Domestic dogs fed raw offal are more likely to test positive for *Echinococcus* spp. coproantigen (Buishi *et al.*, 2006; Moro & Schantz, 2009), and the feeding of infected offal perpetuates *Echinococcus* spp. transmission (Eckert & Deplazes, 2004; Otero-Abad & Torgerson, 2013).

Raw equine meat and offal were also commonly fed to 59.4% and 31.3% of hunts respectively. Transmission of *E. equinus* in the UK is believed to be maintained through a hunting hound-horse lifecycle. Evidence of this feeding practice in this and other studies (Lett, 2013; Lett *et al.*, 2018; Thompson & Smyth, 1975), together with molecular diagnostic evidence of *E. equinus* carriage in UK hunting hounds (Boufana *et al.*, 2015) supports this hypothesis.

It is concerning that the practice of feeding raw offal from fallen stock of any species, classed as category 2 ABP material, is common within the hunts in this study. This practice contravenes the conditions for the feeding of certain category 2 ABP material to hunting hounds in the UK under the current derogation from the law

(DEFRA, 2011b). This issue should be raised and discussed with APHA, the Hunting Office and other stakeholders. Over half of hunts (56.3%) were registered as collectors of fallen stock through the NFSCo scheme. In this study, hunts reporting membership of the scheme were also feeding raw offal from fallen stock, in contravention with the Terms and Conditions of NFSCo membership, which require the collector to abide by legislation concerning the feeding of category 2 ABPs to hounds i.e. to not feed raw offal from category 2 material (NFSCo, 2019b).

The derogation allowing the feeding of category 2 ABP from fallen stock to hunting hounds states that the animal must not have died from a disease transmissible to humans or animals (DEFRA, 2011b). In their review of the biosecurity risks relating to hunting with hounds, Harris and Dorning proposed that, without a routine *post-mortem* examination, it is impossible to know whether fallen stock fed to hounds as category 2 ABP died as a result of an infectious disease (Harris & Dorning, 2017). Between March 2014 and March 2016, The Fallen Stock Project, an AHDB Beef and Lamb-funded project to assess the value of *post-mortem* examination of fallen stock to improve and inform stock health examined over 2,400 carcases of sheep and cattle. The project identified over 100 causes of death in sheep and cattle, although no diagnosis was reached in 15-44% of cases (AHDB, 2016). Hydatidosis in sheep and cattle has been associated with production losses in the UK (AHDB, 2018) but it is not typically a cause of mortality. It is possible that fallen stock may be sub-clinically infected with *E. granulosus* yet have died from a cause that would not preclude feeding of the carcase to hounds. As post-mortem inspection of fallen stock is not mandatory, hydatidosis could go undetected.

The feeding of raw ABP categories according to risk of *Echinococcus* spp. infection varied significantly between hunt types. Foxhound packs were significantly more likely to feed raw ABP in all the risk categories for *Echinococcus* spp. infection, including very high risk for *E. granulosus* and *E. equinus* transmission. Beagles were significantly associated with the feeding of high risk ABP for *Echinococcus* spp. infection. This finding would suggest that some hunt types are at greater risk of *Echinococcus* spp. infection through their feeding practices than others. Studies of UK foxhounds have demonstrated *Echinococcus* spp. infection in packs feeding raw

meat and offal (Boufana *et al.*, 2015; Lett, 2013; Lett *et al.*, 2018). A questionnaire study of 21 hunts of different types in Great Britain reported raw feeding of sheep liver and lungs in 43-47.4% and bovine liver and lungs in 63.2-65% of hunts, depending on hunt type, with little difference between hunt types. In contrast, the same study reported much higher feeding of equine liver and lungs to foxhounds, harriers and staghounds (63%) than to beagles, basset hounds, bloodhounds and other hunt types (21.1%) (Thompson & Smyth, 1975).

While new and existing evidence of the risk of *Echinococcus* spp. transmission through the feeding raw ABP needs to be generally communicated to the hunting community there is scope to impress these risks in particular to hunt types more likely to feed high risk ABP and their member associations.

A study of 12 foxhound packs in Wales reported only finding *E. granulosus* on arecoline purgation of hounds from packs fed raw meat or offal from sheep, cattle or horse and none from those where such food was boiled first (B. M. Williams, 1976). Despite advice in The Code of Practice for the Welfare of Hounds in Kennels that cooking raw meat and offal will reduce the risk of transmitting infectious pathogens including tapeworms and *Salmonella* spp. (The Council of Hunting Associations, 2015), only one hunt in this study was cooking raw ABP prior to feeding to the hounds.

Feeding of proprietary food was common to the majority of hunts (68.8%) in this study, although only 15.6% were feeding this exclusively. The Code of Practice for the Welfare of Hounds recognises that the nutritional and energy needs of hounds can be well met and better regulated by feeding proprietary dog food (The Council of Hunting Associations, 2015). However, as this study, and recent surveys of UK hunting hound diet demonstrate (Lett *et al.*, 2018), the feeding of raw meat and offal from a number of livestock species and sources remains widespread. None of the hunts testing positive for *Echinococcus* spp. were exclusively feeding proprietary dog food.

In the absence of routine post-mortem examinations, there is little information available on the causes of death in fallen stock. Hydatid cysts only typically cause morbidity in the intermediate host when they reach a size or number that impede the function of the organ of origin. Thus, carcases of dead livestock not deemed to have died from an infectious disease and processed as category 2 ABP by hunts as collection centres, may still harbour unseen hydatid lesions.

This study demonstrates that the conditions under which a derogation in law permits the feeding of certain category 2 ABP to hunts are not being met i.e. hunts are continuing to feed offal from fallen stock.

3.4.4. Food storage and disposal

The majority (81.5%) of hunts in this study feeding raw ABP material stored it at room temperature. Carcases or part-carcases fed to hounds are typically processed and stored in indoor spaces referred to as 'flesh rooms' at the hunt kennels, which are typically not artificially refrigerated. None of the food storage methods used in this study, even conventional freezing at -20^{o}C, would be sufficient to kill the infective larval stage of *Echinococcus* spp. if present in the raw meat source, unless stored for a minimum of one week at this temperature (ESCCAP, 2017). Although details fall outside the remit of this study, this practice has implications for microbiological food safety, as such storage could promote the growth of bacteria pathogenic to humans and animals (Harris & Dorning, 2017). Furthermore, open storage of this type could encourage vermin to the site, presenting a further possible risk to public and animal health. The Code of Practice for the Welfare of Hounds in Hunt Kennels recommends the storage of raw flesh in a 'cold room' but other than recommending hosing of the space after use, it does not specify how conditions could prevent raw food spoilage or further mitigate the risk of food-borne disease (The Council of Hunting Associations, 2015).

Hunts must dispose of ABP waste via an approved processing or incinerating facility (DEFRA, 2014). The majority of hunts in the study did so either via a rendering plant (82.6%) or an incinerator facility (13%). The finding that 17.4% of hunts reported

disposing of ABP by returning it to the supplier is contradictory to current legislation. ABP sourced from an abattoir or as fallen stock from a farm would not be disposed of via return to these locations. However, a situation may arise where, for example: Hunt A is a registered collection centre, final user and incineration facility for ABP; Hunt B is registered as a final user only. Hunt A may process and provide category 2 ABP to hunt B, who then returns unused and waste ABP to Hunt A for disposal. It is not known how many respondents in this case are in such a scenario (although no hunts reported having an on-site incinerator themselves), how many are engaging in unlawful practices or how many may have misunderstood the question.

3.4.5. Parasite control and prevention

All participating hunts reported administering anthelmintic products to the hounds on a regular basis as part of a Hound Health Programme; a requirement under the Code of Practice for the Welfare of Hunting Hounds, issued by the Council of Hunt Associations, which currently recommends worming with a praziquantel wormer twice yearly, at the start and end of the hunting season (The Council of Hunting Associations, 2015).

This study found that 44.3% of participating hunts were not using a praziquantel-based wormer. This is a lower proportion than the 56% of hunts reported as not using praziquantel in a survey of 16 foxhound packs in England and Wales (Craig *et al.*, 2012; Lett, 2013). The study also examined the dosing and frequency of the 66.7% of hunts that did use praziquantel and found that only approximately a quarter of these (27.8%) were doing so at a minimum protocol (minimum 4 times yearly) to reduce *Echinococcus* spp. infection. The results suggest that knowledge of, or a willingness to adopt, optimal worming strategies for the prevention of *Echinococcus* spp. infection is lacking.

Notably, all participating hunts in Wales, where Government-led hydatid control schemes have been implemented in the past, were administering a praziquantel wormer. In contrast, Northern Ireland, where *E. granulosus* is not endemic, was the only country where no participating hunts were administering praziquantel.

An association between hunt type and use of a wormer containing praziquantel was close to statistical significance (p=0.055). When analysed individually, Harrier packs were significantly more likely to administer a worming product that did not contain praziquantel ($p<0.05$). Although the sample size may have affected the results, this would be important information to relay to the Association of Masters of Harriers and Beagles.

Of the nine licensed wormers used by participating hunts, only six contained praziquantel. Most praziquantel wormers in use were available without a veterinary prescription. Aside from licensed product, hunts were also administering a variety of worming products empirically that were not licensed for use in dogs. In discussion with the author at a county show in 2017, a kennel huntsman described how many hunts avoided seeking veterinary advice on hound health matters, largely due to the cost of advice and treatments, preferring instead to address health issues among themselves (Anon. pers. comms). Although this is not likely to represent the actions of the hunting community as a whole, the finding in this study of widespread use of wormer preparations not licensed for use in dogs suggests veterinary input on worming advice is suboptimal. The empirical use of ivermectin and moxidectin-based products designed for large animal use does not protect against *Echinococcus* infection and has a narrow margin of safety when used in dogs (NOAH, 2017).

Two out of four hunts testing positive for *Echinococcus* spp., including the hunt testing positive for *E. granulosus* (G1) were using a praziquantel wormer, however neither were doing so at the minimum or optimal recommended doses for *Echinococcus* prevention. This highlights the importance of adequate dosing of praziquantel when given, if it is to be effective in preventing *Echinococcus* infection and transmission. Praziquantel does not have sustained activity following dosing, so reinfection post-dosing is possible if there is continued exposure through the feeding of infected meat and offal. The current guidelines of twice-yearly worming with praziquantel recommended by the Council of Hunt Associations (The Council of Hunting Associations, 2015) are inadequate for the prevention and control of *Echinococcus* spp. transmission. Annual or bi-annual treatments have no significant

impact on preventing patent infection with common helminths and cestodes in dogs (Sager *et al.*, 2006).

Current ESCCAP guidelines on anthelmintic dosing effective against *Echinococcus* are given on a risk-based approach. Hunting hounds regularly fed raw offal as part of their diet are recommended to be routinely wormed with a praziquantel wormer every 6 weeks to prevent infection and egg shedding (ESCCAP, 2017). No hunts in this study had adopted this risk-based approach to worming against *Echinococcus* spp.

3.4.6. Land access and faeces disposal

While hounds are typically not fed for up to 24 hours before a hunt, gut transit times mean that they are still likely to defecate on the day of the hunt (Harris & Dorning, 2017). As discussed in the introduction of this chapter, the collection of faeces voided by the hounds while they are engaged in a hunt would not be possible.

All participating hunts reported regular removal and disposal of faeces voided at the kennels, although it is not clear whether this also included any enclosed areas used for exercise. Fenced concrete and grass yards were commonly used by over half of hunts in the study to exercise the hounds. Several cases of severe transdermal infection of the hookworm *Uncinaria stenocephala* into the feet of foxhounds in a UK hunt demonstrate the risk of accumulated parasitic contamination in grass yards used to exercise hounds (Barker, 2010).

The most common means of faeces disposal was onto a muck heap, a method used by over a third (37.5%) of hunts. Together with the use of slurry pits or disposal directly onto farmer's fields, the study found that half of participating hunts were disposing of hound faeces in ways that could potentially contaminate agricultural or horticultural land. Under temperate conditions such as those in the UK, *Echinococcus* eggs can remain viable in the environment for over 1 year, posing a sustained infection risk to livestock if untreated dog faeces are spread on grazed land (Craig *et al.*, 2015). There are no specific studies addressing the effect of composting or sewage sludging on the viability of *Echinococcus* eggs (Eckert *et al.*, 2001).

It was common among participating hunts to exercise hounds on land with shared access to livestock (62.5%) and open land with public access (68.8%). Although the questionnaire did not specifically ask about hound access to land used by horses, Thompson and Smyth (1975) reported shared use of fields and paddocks between hounds and a hunt's horses as a risk factor for *E. equinus* transmission (Thompson & Smyth, 1975).

Exercising activities can vary depending on the age of the hounds. Every hunt typically has a group of 'puppy walkers' whose responsibility it is to raise pups by offering a temporary home for their first six months after weaning. Puppy walkers are selected from applicants by the hunt and are actively encouraged by the hunt to lead train the pups and introduce and habituate them around livestock, other dogs and people (Austen, 2014). While on-lead walks are likely to facilitate the collection and disposal of hound pup faeces, this period of increased contact with livestock and people could present an *Echinococcus* infection risk if the pups are fed raw meat and offal and are not appropriately wormed. To the author's knowledge, studies of *Echinococcus* carriage in hunting hound pups and the associated risks of transmission during this period of increased human and livestock contact have not been undertaken. Exercising the main pack can involve walking out with the hounds, exercising on roads or tracks (termed 'roading') or running the pack with riders on horseback (Dangar, 1994). The land used for exercising outside of the hunt season is typically the same as that used for the hunt meets. As such, the likely risk of *Echinococcus* transmission to grazing animals associated with hound packs defecating on pastures and along roads and tracks used by livestock would not be limited to the hunting season (Harris & Dorning, 2017). The practice of 'billeting' hounds to farms outside of the hunting season may also contribute to potential faecal contamination on-farm and may give hounds the opportunity to scavenge fallen stock carcases (Boufana *et al.*, 2015).

3.4.7. Conclusions

This study has undertaken a UK-wide investigation of *E. granulosus* in hunting hound packs using a combined faecal sampling and questionnaire survey. The study aimed

to identify *Echinococcus* spp. infection at the hunt level and investigate associated risk factors for infection. In agreement with the literature, the findings suggest that *Echinococcus* spp. including zoonotic *E. granulosus* are circulating between UK hunting hounds and intermediate livestock and equine hosts. The study has identified *Echinococcus* spp. infection in hounds by coproELISA and coproPCR in previously unreported regions in the UK, including Scotland, the North and South West of England. This finding supports the overarching hypothesis of the thesis that echinococcosis, and in particular *E. granulosus,* is more widespread in the UK than previously understood. Confirmatory species-specific coproPCR and sequencing of samples testing positive on coproELISA would support this further. As with the other canid studies, challenges can exist with the efficacy of coproELISA and coproPCR tests which can affect test sensitivity, especially in low prevalence settings. It is possible that underrerporting may have occurred as a result.

Questionnaire responses identify a lack of adequate dosing of hunt packs with praziquantel-based wormers. Alongside evidence of widespread feeding of raw meat and offal from fallen stock, there is a viable and sustained risk of transmission of zoonotic echinococcosis in this population. This study highlights the need to communicate these risks and the importance of risk-based measures to mitigate them. However, lessons from the evaluation of *Echinococcus* control programmes worldwide recognise the value in selecting workable control targets in a given setting (Craig *et al.*, 2017). The logistics and costs involved with 6-weekly worming of a hunt pack with praziquantel would make it unlikely that hunts would adopt this optimal protocol, nor would they readily stop the feeding of fallen stock to hounds. The collection of fallen stock by hunts offers a source of income and provides a service to farmers who reciprocally permit hunts onto their land. Implementing realistic and workable changes to husbandry practices in UK hunts that aim to reduce *Echinococcus* transmission risk is a difficult but important target. Success will need the trust and cooperation of the hunting community, their representing associations and the veterinary practices attending to them.

Chapter Four

Echinococcus spp. in canids and hyaenids in UK zoos

4 *Echinococcus spp. in canids and hyaenids in UK zoos*

4.1 Introduction

Many wild canid and hyaenid species are competent definitive hosts of gastrointestinal (GI) parasites of public health importance (Lim *et al.*, 2008; Opara, Osuji & Opara, 2010; Williams & Thorne, 1996), including zoonotic *Taenia* and *Echinococcus* tapeworm species (Panayotova-Pencheva, 2013a; Ramos, 2014; Sobrino *et al.*, 2006). A recent review of parasite carriage in captive collections in European zoos reports helminths and cestodes as the first and second most frequently described parasites (Panayotova-Pencheva, 2013b). This poses a potential transmission risk to animals and humans from species such as wolves, painted dogs, foxes and coyotes, which are common to publicly accessible UK zoo collections. These animals are fed raw meat in their diet, including whole and part-carcases of livestock, to meet their natural nutritional and behavioural needs (Hüttner *et al.*, 2009).

As part of licensing and accreditation, UK zoos and wildlife parks must have protocols and measures to treat GI parasite infections in their collections and reduce any associated risk to protect visitors and staff (BIAZA, 2019; Great Britain., 1981). However, research to better understand whether such measures adequately address the risks to animals and humans from zoonoses such as *E. granulosus* in this setting are lacking. Recent reports of fatal cystic echinococcosis in captive-bred non-human primates and ungulates in UK zoos raise concern about *Echinococcus* circulating in this setting (Boufana *et al.*, 2012a; Denk *et al.*,, 2016).

This chapter introduces the epidemiology and ecology of *Echinococcus spp*. in species common to zoo collections, focusing on case reports of infection in UK zoo populations, alongside the legislation and management designed to mitigate zoonotic infection risk. It includes the methodology and results of the survey, conducted in collaboration with the British and Irish Association of Zoos and Aquariums (BIAZA), together with a reflection on the findings and their implications for animal and public health in UK zoo settings.

4.1.1. Epidemiology of *Echinococcus spp*. in wild and captive canid species

4.1.1.1 Echinococcus spp. infecting wild canids and hyaenids common to UK zoo collections

E. multilocularis, *E. vogeli*, *E. canadensis (G8-G10)*, *E. shiquicus*, *E. oligarthus* and *E. felidis* are transmitted through sylvatic cycles worldwide (Romig *et al.*, 2015). Foxes, wolves, jackals and raccoon dogs have a role in the transmission of *E. multilocularis* in endemic parts of Europe, through predominantly sylvatic cycles, involving rodents as intermediate hosts (Carmena & Cardona, 2014). Bush Dogs are the main definitive host for *E. vogeli*, the cause of polycystic echinococcosis, a neglected zoonotic disease in South America (do Carmo Pereira Soares *et al.*, 2014; Manter, Rausch, & Bernstein, 1972; Moro & Schantz, 2009). Conversely, *E. granulosus*, *E. equinus*, *E. ortelppi* and *E. canadensis* (G7-G9) are largely transmitted through domestic and synanthropic cycles (Romig *et al.*, 2017, 2015). However, in contrast to their sylvatic counterparts, there is evidence that many canid species kept in zoos are competent hosts of *Echinococcus spp.*, transmitted through domestic cycles, such as *E. granulosus* and *E. equinus* (Table 4-1).

Hyaenid species have been identified as competent hosts of *Echinococcus* spp., however they are thought to play a minor role as a definitive host due to a reduced susceptibility to infection and small wildlife populations (Carmena & Cardona, 2014).

In Europe, wolves are the only species to have been identified as a definitive host of *E. granulosus s.l.* to date (Carmena & Cardona, 2014). Surveillance studies in wild fox populations in the UK and Ireland have not demonstrated the presence of *E. multilocularis* (Learmount *et al.*, 2012; Murphy *et al.*, 2012), leading to the UK and Ireland being declared free of *E. multilocularis* (Torgerson & Craig, 2009).

4.1.1.2 Risk factors for Echinococcus spp. infection in captive definitive and intermediate hosts

Zoos, safari parks and private wild animal collections house multiple species within a restricted controlled environment. This specific captive animal/wildlife/human interface could increase the risk of zoonotic disease spread to zoo personnel and

visitors and facilitate parasite transmission between captive species and native wildlife (Warwick *et al.*, 2012).

Table 4-1. Examples of Echinococcus spp. in wild and captive canid and hyaenid species common to zoo collections. E. granulosus sensu lato (s.l) refers to the species complex of Echinococcus responsible for causing CE.

Species	Common name	*Echinococcus* species	Reference
Lycaon pictus	African Hunting Dog	*E. granulosus s.l.*	Hüttner and Romig, (2009); Kagendo *et al.*, (2014)
Otocyon megalotis	Bat-eared Fox	*Echinococcus spp.*	Ernest, Nonga and Cleaveland, (1979)
Canis mesomelas	Black-backed Jackal	*E. equinus* *E. granulosus s.l*	Wassermann *et al.*, (2015) Macpherson *et al.*, (1983)
Speothos venaticus	Bush Dog	*E. vogeli*	do Carmo Pereira Soares *et al.*, (2014)
Vulpes corsac	Corsac Fox	*E. multilocularis*	Tang *et al.*, (2006)
Canis lupus lupus	European Grey Wolf	*E. granulosus s.l* *E. granulosus s.s*	Shimalov & Shimalov, (2000) Sobrino *et al.*, (2006)
Canis lupus signatus	Iberian Wolf	*E. granulosus s.l* *E. intermedius*	Sobrino *et al.*, (2006) Guerra *et al.*, (2013)
Vulpes vulpes	Red Fox	*E. granulosus s.l* *E. granulosus s.l*	Dalimi. A, (2010) Jenkins *et al.*, (2000)
Crocuta crocuta	Spotted Hyena	*E. felidis* *E. granulosus s.l*	Owerri, (2010) Lahmar *et al.*, (2009)
Canis familiaris dingo	Dingo	*E. granulosus s.l*	Jenkins and Morris, (2003)
Lycaon pictus	African Hunting Dog	*E. granulosus s.l*	Hüttner and Romig, (2009); Kagendo *et al.*, (2014)
Otocyon megalotis	Bat-eared Fox	*Echinococcus spp.*	Ernest, Nonga and Cleaveland, (1979)
Canis mesomelas	Black-backed Jackal	*E. equinus*	Wassermann *et al.*, (2015)
Vulpes lagopus	Arctic Fox	*E. granulosus s.l* *E. multilocularis*	Macpherson *et al.*, (1983) Stien *et al.*, (2010)

Captive carnivore species are commonly fed a diet including raw meat and offal from part or whole carcases (see section 4.1.2.2 for further details on feeding of ABP to zoo animals). *E. granulosus* and *E. equinus* are both endemic to the UK and could potentially transmit to competent captive canid species through the feeding of infected raw livestock products. Foods such as fruits, vegetables and cereal crop fodder brought in for animal collections have been suggested as potential routes of *Echinococcus* spp. introduction to infect intermediate hosts in UK zoos (Boufana *et al.*, 2012b; Denk *et al.*, 2016).

Zoos are important for the conservation of endangered species. The movement of animals between zoos, and often between countries, to augment collections or assist breeding programmes could also present possible routes for introduction of parasitic infections (Warwick *et al.*, 2012). Legal importation of captive wild mammals into zoo or private collections and illegal importation of wild or domestic mammals have been identified as potential routes for the introduction of *E. multilocularis* into the UK (Torgerson & Craig, 2009).

Captive definitive hosts in zoos and wildlife parks may come into contact with native wildlife. A study of *E. multilocularis* in a wildlife park in France reported infection in captive wolves through consumption of wild rodents within their enclosures. The study demonstrated that perimeter fencing was inadequate in keeping out wild foxes infected with *E.multilocularis* (Umhang *et al.*, 2016). A further study of cestodes including *E. granulosus* and *E. multilocularis* in captive non-human primates in France suggested the existence of transmission at the captive animal-native wildlife interface within a primate centre (Greigert *et al.*, 2019).

4.1.1.3 Public health risks relating to zoonotic Echinococcus spp. in zoos

Maintaining captive wild animal collections, whether open to the public or not, presents potential zoonotic disease risks. It has been estimated that 75% of emerging infectious diseases are zoonotic, with 70% of these originating within wildlife populations (Jones *et al.*, 2008). Zoos present a unique interface between substantial populations of captive species, humans and native wildlife, which merit specific biosecurity protocols to mitigate the introduction and spread of pathogens (Stirling *et al.*, 2007).

The degree of animal-human contact in zoos will vary depending on several factors, including the type of establishment (e.g. walk-through zoo, drive-through park, petting zoo), the animal species and their specific husbandry needs, the level of animal domestication and any activities that actively encourage human contact. Traditional zoos do not generally pose the same health risks as open farms and petting zoos due to reduced contact between animals and visitors (DEFRA, 2012c).

Typically, visitors to zoos would not have contact with captive carnivores for health and safety reasons. However, a number of UK zoos and wildlife parks offer guided encounters to observe and feed species such as wolves, or 'zookeeper for a day' experiences that bring the public in closer contact with such species. The training of animals in zoos to facilitate routine healthcare and husbandry procedures by zoo personnel, such as administration of medication and veterinary checks, is an increasingly common practice (European Commission, 2015). Zoo keepers and veterinary staff will be in regular contact with animal urine and faeces during enclosure cleaning and disinfection.

High profile outbreaks of zoonotic disease at animal visitor attractions, such as an outbreak *Cryptosporidium parvum* in visitors to a petting farm in Surrey (Utsi *et al.*, 2019) and *Shigella flexneri* in an animal keeper and a primate collection in a zoo in Vienna (Lederer *et al.*, 2005) highlight disease transmission risks in these settings.

Given that zoonotic *Echinococcus spp.* transmission to humans is primarily though accidental ingestion of eggs on canid fur or in faeces (Eckert & Deplazes, 2004a; Moro & Schantz, 2009; Torgerson & Budke, 2003), it is likely to be zoo keepers and veterinary staff who are at greatest risk of infection in collections housing canids and hyaenids. The Health and Safety Executive (HSE), the UK government agency responsible for workplace health, safety and welfare, lists veterinary surgeons, dog handlers, waste disposal workers and others in contact with infected dogs or faeces from infected dogs as at risk of occupationally-acquired hydatid disease (Health and Safety Executive, 2015). Risk assessments for any capture, handling or transport of captive wild animals and maintenance of their enclosures should include the risk of potential zoonotic disease infection (BIAZA, 2019).

A study of employees at a zoo in Austria screening for antibodies to selected zoonotic agents found 97% of individuals seropositive for at least one zoonotic pathogen. However, in the study it was not possible to establish causal links between seropositivity and activities at the zoo, nor were any individuals seropositive for *E. granulosus* or *E. multilocularis* (Juncker-Voss *et al.*, 2004). A review of occupational zoonoses in zoo and wildlife veterinarians in India concluded that regular screening

of zoo staff for zoonotic diseases should be a part of occupational health and safety strategy (Chethan-Kumar *et al.*, 2013).

4.1.1.4 Echinococcus spp. in captive mammals in UK zoos

Zoo collections can house intermediate hosts of *Echinococcus* spp., including ungulates, rodents and non-human primates (Romig *et al.*, 2017). Several *Echinococcus* species, including *Echinococcus granulosus* G1 and *Echinococcus multilocularis* have been identified at post-mortem in captive mammals in UK zoos and wildlife parks. In a published case series, Boufana *et al.* reported six cases of larval infections of *Echinococcus spp.* in captive mammals from UK collections. They identified by post-mortem and confirmed by molecular genotyping *E. granulosus* G1 in a Philippine spotted deer (*Rusa alfredi*) imported from France and a Red-tailed guenon (*Cercopithecus ascanius*) imported from Israel and *E. ortleppi* in a second Philippine spotted deer from France. The same group identified *E. multilocularis* in a Barbary macaque (*Macaca Sylvanus*) imported from Germany and *E. equinus* in a Burchell's zebra (*Equus burchellii*) and a red ruffed lemur (*Varecia rubra*). Both cases of *E. equinus* were captive born in the UK and had not been moved elsewhere (Boufana *et al.*, 2012). A further study reported the first case of *E. ortleppi* (G5) in a captive-born lemur in the UK. The case had a history of an escape from the zoo collection into a nearby village, where it was recaptured (Denk *et al.*, 2016).

The first report of *E. multilocularis* in an animal in Great Britain, a translocated Eurasian beaver (Barlow, Gottstein, & Mueller, 2011) imported as part of a reintroduction programme to the UK, prompted a DEFRA-led qualitative risk assessment of the likelihood of introduction of the parasite from escaped or released individuals of the species (Roberts, 2012).

4.1.1.5 Cestode treatment and prevention in UK zoo canids

Regular worming under veterinary guidance as part of preventive medicine and disease control is stipulated in legislative and guideline documents, applicable to UK zoos and wildlife parks (BIAZA, 2014, 2019; Council of the European Union, 1992,

1999; DEFRA, 2012b; EAZA, 2014; Great Britain., 1981; Health and Safety Executive., 2012).

Common helminth parasites in wild canids include roundworms (ascarids), hookworms, whipworms and tapeworms (Prociv & Cross, 2001). Standard anthelmintics at domestic dog doses have been successfully used to treat internal parasites in zoo canids (AZA, 2012a). Praziquantel has been recommended for treatment and prevention of cestode infections, including *Echinococcus spp.* in large canids at the recommended dose for domestic dogs (Grisham *et al.*, 1994; Mcruer & Barron, 2018). For all other common intestinal helminths (including *Taenia spp.* but not *Echinococcus spp.*), fenbendazole, pyrantel or ivermectin have been recommended (AZA, 2012a; Grisham *et al.*, 1994). Some combination wormer preparations licensed for domestic dog use are only available through veterinary prescription (POM-V). Such wormers are used 'off-label' for non-domestic canid and hyaenid species under the prescribing cascade. If there is no authorised veterinary medicine available in the UK suitable for a purpose in a given species, the cascade system permits a veterinary surgeon to prescribe a product licensed for the same purpose in another species (VMD, 2015).

4.1.2. Regulation of zoos and wild animal collections in UK

4.1.2.1. Legislation governing UK zoo collections

The Zoo Licensing Act (1981) for England, Scotland and Wales (Great Britain., 1981), and the Zoo Licensing Regulations in Northern Ireland (Great Britain., 2003) are the primary legislation for the licensing and inspection of zoos in the UK. Wild animal collections that are open to the public for more than 7 days in a 12-month period and containing typically more than 120 animals require licensing under the Act. The Zoo Licensing Act is supplemented by the Secretary of State's Standards of Modern Zoo Practice, which guides on the Act's requirements and implementation (DEFRA, 2012b). In 2003, the Act was amended to introduce the provisions of the European Zoos Directive (Council Directive 1999/22/EC). The focus of the Act is to uphold standards of animal welfare and public safety and the focus of the Directive is conservation, education and research. Where standards are not met, local

authorities can place conditions on zoo premises, action amendments and withdraw zoo licenses in the event of non-compliance.

Other private collections housing certain wildlife species that do not qualify under the Zoo License Act require registration under The Dangerous Wild Animals Act (1976) (Great Britain, 1976) and will be subject to inspections by their local authority, under guidance of APHA.

Inspections of zoo premises to obtain or renew a license are undertaken at the local authority level by zoo specialists (vets and zoo managers) appointed by APHA. Both the Act and the Directive make provisions for preventative healthcare that include parasite control, feeding practices that meet nutritional and behavioural needs and control of pests and vermin. The HSE have issued practical guidance in conjunction with the Zoo Licensing Act to encourage best practice in managing health and safety in zoos (Health and Safety Executive., 2012).

Movement of non-domestic carnivores intended for display or conservation between the UK from other EU or non-EU countries must be in accordance with the Balai Directive (Council Directive 92/65/EEC, 1992). Animal movement under the Directive can only occur between premises approved and registered by authorities in the country of origin. Under the directive, UK zoos must be inspected and approved by APHA on behalf of DEFRA, the Scottish Government and Welsh Government and by DAERA in Northern Ireland. Currently, there is no provision within the Directive to treat or test non-domestic carnivore species, including competent hosts for zoonotic *Echinococcus spp.*, with a praziquantel wormer prior to movement (APHA Centre for International Trade, pers. comm.). In contrast, under the current Pet Travel Scheme, domestic dogs entering the UK from most EU and non-EU countries are required to receive treatment with a praziquantel wormer 1-5 days prior to UK entry to prevent importing *E. multilocularis* (DEFRA, 2019).

4.1.2.2. The British and Irish Association of Zoos and Aquariums (BIAZA)

This study was undertaken in collaboration with BIAZA, a charity organisation and professional body representing over 100 zoos and aquariums in Britain and Ireland.

BIAZA supports the conservation, education and research activities of its member organisations via several committees and working groups. Member organisations must meet the requirements of membership as detailed in the BIAZA's policy documents, which include maintaining a 'high standard of husbandry with a strong programme of veterinary medical care at both preventive and curative levels' (BIAZA, 2019). Member organisations must comply with all relevant regulations and legislation (see section 4.1.2) and share with BIAZA the outcome of inspections under zoo licensing laws.

The BIAZA Research Committee offer guidelines for conducting research in zoos and aquaria (Bishop, Hosey, & Plowman, 2015) and will consider applications for support of research projects conducted within member collections. Obtaining BIAZA research support encourages zoos to take part in research that has been identified by BIAZA as relevant to animals in their care and to the wider zoo community. The BIAZA Research Committee also holds annual zoo research symposia for academics, students and zoo professionals.

4.1.2.3. Feeding of ABP to zoo animals

The general EU legislative framework currently in place in the UK relating to the use of ABP as animal feed is summarized in Chapter 1. In brief, at the time of writing, EU Regulation (EC) 1069/2009 and its implementing Regulation (EC) 142/2011 allows the collection and use of Category 3 ABP, the lowest risk category, for the manufacture of pet food.

Alongside hunt kennels, zoos are able to register with APHA in England, Scotland and Wales or DAERA in Northern Ireland as collection centres of ABP from fallen stock or as final users of ABP from abattoirs or other collection centres. Two derogations exist under Regulation (EC) 1774/2002 that may be adopted by devolved UK governments acting as competent authorities. Firstly, the feeding of Category 3 and Category 2 ABP materials to zoo animals, provided Category 2 materials are not from animals that were killed or died from disease posing a risk to human or animal health. Secondly, a derogation permits zoo animal carcases, classed as Category 1 ABP

material to be fed to other zoo animals, where there is no known risk of disease transmission. In the UK, these derogations have been adopted by the devolved administrations in England (DEFRA, 2011), Scotland (Scottish Government., 2014), Wales (Welsh Assembly Government, 2011) and Northern Ireland (Northern Ireland Government, DAERA, 2014). Unlike hunting hound packs, zoos are not able to register as collection centres within the NFSCo Scheme.

4.1.3. Study aims

Many zoos in the UK house carnivores that are competent hosts for zoonotic *Echinococcus spp.* and may infect other animals, such as non-human primates and ungulates, common to zoo collections. Previous studies have diagnosed cases of autochthonous fatal cystic echinococcosis in intermediate hosts in UK zoos (Boufana *et al.*, 2012). The risks and pathways of *Echinococcus* transmission in zoos and the potential role of canid and hyaenid collections are poorly understood. There is a lack of research investigating the prevalence and importance of echinococcosis in UK captive canid species and the implications to animal and public health.

The primary aim of this study was to asses the proportion of canid and hyaenid species in participating UK zoo collections testing positive for *Echinococcus* spp. at the genus and species level. The study also aimed to identify potential risk factors associated with the transmission dynamics of *Echinococcus* spp. including diets fed, worming practices, extent of collection and disposal of faeces and routine cestode parasite testing at the species enclosure and zoo level. As part of the broader thesis aim, the study aimed to contribute towards a greater understanding of *Echinococcus* spp. distribution in the UK to better inform surveillance, public health information and future control efforts.

4.2. Methodology

The study methodology follows the questionnaire, faecal sampling and laboratory approach described in Chapter 2. Any steps specific to the study design and recruitment process are described in detail here.

4.2.1. Study design

The study was undertaken with support from BIAZA and in collaboration with zoos and wildlife parks in the UK and Ireland. Inclusion criteria for the study were BIAZA membership by zoo collections in the UK and Ireland (BIAZA, 2019) holding carnivores within the families *Canidae* and *Hyaenidae*. Up-to-date species holdings at all eligible zoos and wildlife parks in UK and Ireland were obtained by request from the Zoological Information Management System (ZIMS), the largest global database of wildlife species holdings, curated by Species360, a global information network of wildlife care and conservation institutions (ZIMS, 2019). Eligible UK zoos and wildlife parks were also crosschecked on the current APHA database of premises approved for the collection and/or use of ABP (APHA, 2018a). The species of interest held by BIAZA member collections in the UK and Ireland included: African Hunting Dog (*Lycaon pictus),* Bat-eared Fox (*Otocyon megalotis),* Bush Dog (*Speothos venaticus),* Black-Backed Jackal (*Canis mesomelas),* Coyote *(Canis latrans)* Corsac Fox (*Vulpes corsac),* Eastern Aardwolf (*Proteles cristata),* European Grey Wolf (*Canis lupus lupus),* White wolves *(Canis lupus arctos),* Arctic Fox *(Vulpes lagopus),* Red Fox *(Vulpes Vulpes),* Fennec Fox (*Vulpes zerda),* Iberian Wolf (*Canis lupus signatus),* Maned Wolf (*Chrysocyon brachyurus),* Dingoes *(Canis lupus dingo),* Singing Dog (*Canis lupus dingo hallostromi*), Spotted Hyena (*Crocuta crocuta)* and Dhole (*Cuon alpinus*).

Ethical approval (Ref. VREC603) was granted by the University of Liverpool Veterinary Research Ethics Committee in January 2018 and sampling commenced in February 2018. The study was jointly conducted with a University of Liverpool Masters student, Elizabeth Attree, who was undertaking a coprological survey of parasites in zoo canid faeces. In January 2018, the study was successful in obtaining research support from the BIAZA Research Committee and a letter of support from BIAZA was issued in February 2018 encouraging member zoos to take part (Appendix II-a). In order to encourage participation, zoos remained anonymous in any study outputs. Study recruitment and sample collection was completed in May 2018.

A study questionnaire, consent form and participant information sheet (Appendix II-b, II-c) were designed using Microsoft Word 2010 (Microsoft Corporation, USA). A

separate questionnaire and sampling kit were provided for each relevant canid or hyaenid species kept at a participating zoo. The questionnaire, for completion by the attending keepers and/or zoo veterinarians, included sections on housing and husbandry, types and origin of diets fed, worming and faeces disposal, following the general format as described in Chapter 2. Further study-specific modifications to the questionnaire included canid/hyaenid species and number kept, movement of animals between collections and routine testing for faecal parasites. The questionnaire format required mostly tick-box responses, with occasional open-ended questions where appropriate. The questionnaire and supporting documents were reviewed by the BIAZA Research Committee as part of the successful application for BIAZA project support.

Zoos and safari parks were contacted by the lead researcher and the MRes student by telephone or email to give details about the study and invite participation. A sampling kit (as described in Chapter 2), consent form and questionnaire were sent by Royal Mail to premises wishing to take part. All samples of voided faeces were collected by zoo staff during routine enclosure management and cleaning, limiting disturbance to animals and ensuring minimal impact on time and resources. Samples were pooled from 6-8 different faecal pats, depending on the number of animals in the enclosure, and unless an animal was housed in isolation, the individual identity of the animals sampled was not known. For this reason, the sampling unit was the enclosure housing the animal group. A separate questionnaire and sampling kit were sent for each species enclosure of interest in a participating zoo. Questionnaires and faecal samples were returned by First Class Royal Mail post in accordance with UN3373 Sample Transport Compliance Regulations. A detailed description of the sampling kit and postal arrangements is described in Chapter 2.

To describe proportions of species enclosures within individual premises and proportions of zoos within the study group engaging in the husbandry and healthcare practices studied, data are presented as proportion, percentage and 95% Confidence Interval (95%CI). A two-sided Fisher's exact test was used to investigate association between coprological test positivity at the species enclosure and zoo level and potential risk factors for infection, with significance set at $p<0.05$. Due to the low

number of positive samples on coproPCR testing, it was not possible to undertake analysis of level of agreement between coproELISA and coproPCR test results. Analysis and graphical display of coproELISA data was performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA). All statistical analyses were performed using Stata 14 (StataCorp, 2015).

4.2.2. CoproELISA

Chapter 2 describes in detail the laboratory protocol of the coproELISA test conducted in this study. In brief, *Echinococcus* spp. coproantigen in zoo canid faeces supernatants was identified using an internationally recognized polyclonal genus-specific *Echinococcus* spp. coproELISA based on the methods of Allan *et al.* (1991) and Craig *et al.* (1995), further optimised at the University of Salford by van Kesteren (2015).

An enclosure was classified as coproELISA positive if one or more single or pooled faecal samples submitted gave a mean coproELISA OD reading (to 3 significant figures) above the defined cut-off value of 0.1221 OD at 620nm. This cut-off value was calculated using a panel of 21 known negative faecal samples from domestic dogs (selected from those previously included in the Gaussian negative panel assay described in Chapter 2) ran in triplicate using the same conditions and reagents. The cut-off value of 0.1221 represents the mean of the replicate ODs plus 3 standard deviations.

Known *Echinococcus* positive and negative faecal samples from the species of interest were not available to this study. As a proxy for species-specific controls, negative samples from UK pet dogs recently wormed with praziquantel and positive control samples from two known *E. granulosus* G1 infected dogs in Kyrgyzstan, confirmed by purgation or coproELISA in a previous study (van Kesteren *et al.*, 2013, Professor M.Rogan, personal communication), or negative faecal samples 'spiked' with *E. granulosus* G1 whole worm extract, were used.

4.2.3. CoproPCR

Chapter 2 describes in detail the coproPCR protocols used in this study. In brief, coproDNA was extracted from zoo canid and hyaenid faeces samples using the QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions for large volumes of starting faecal material. The presence of genus-specific *Taenia* spp. and *Echinococcus* spp. coproDNA was investigated using the multiplex protocol described by Trachsel *et al.* (2007). The protocol by Abbasi *et al.* (2003) was used to identify *E. granulosus* s.l. coproDNA. Species-specific *E. granulosus* G1 and *E. equinus* coproDNA were investigated using protocols by Boufana *et al.* (2013) and Lett *et al.* (2018) respectively. PCR amplification products were identified using agarose gel electrophoresis and where possible, products were sequenced for further species confirmation (Source Bioscience, UK).

4.3. Results

4.3.1. Study response and participation

At the time of study recruitment, there were 120 member zoos and wildlife parks registered with BIAZA in UK and Ireland. The study identified and approached 43 zoos and wildlife parks in the UK housing relevant species of interest. All premises were listed as collection centres and/or final users of ABP with APHA (APHA, 2018).

A total of 22 zoos and wildlife parks agreed to participate in the study, giving an overall participation rate of 51.2% (22/43, 95%CI: 36.2-66.1). The majority of participating zoos were located in England (n=20), with the remainder located in Scotland (n=2). No zoos in Wales or Ireland participated in the study. Two participating zoos in England submitted faecal samples from relevant species holdings but did not submit accompanying completed questionnaires. The results for these two zoos are included in the reporting of coprodiagnostic test results but are omitted from any variable association analyses.

4.3.2. Study species

Responses from the 22 participating premises included samples and information on 41 enclosures (n) representing 12 canid and 2 hyaenid species. The species most commonly represented in the study were the European Grey Wolf (*Canis lupus lupus*) (n=11), Red Fox *(Vulpes Vulpes*) (n=5), Maned Wolf (*Chrysocyon brachyurus*) (n=4) and African Hunting Dog (*Lycaon pictus*) (n=4); other species represented were Bat-eared Fox (*Otocyon megalotis*) (n=2), Bush Dog (*Speothos venaticus*) (n=2), Black-Backed Jackal (*Canis mesomelas*) (n=2), Corsac Fox (*Vulpes corsac*) (n=2), Eastern Aardwolf (*Proteles cristata*) (n=1), Arctic Fox *(Vulpes lagopus*) (n=1), Fennec Fox (*Vulpes zerda*) (n=3), Iberian Wolf (*Canis lupus signatus*) (n=3), Spotted Hyena (*Crocuta Crocuta*) (n=1) and Dhole (*Cuon alpinus*) (n=1).

A summary of species sampled per participating zoo, number of animals per enclosure and numbers of males, females and pups is show in Table 4-2. The total number of males (n=80) and females (n=77) across all participating collections was approximately equal.

In all participating premises, species of interest were kept in single-species enclosures. Only one zoo in England reported receiving individuals from another zoo collection into three out of four of its participating enclosures within the 12 months prior to sampling. The site(s) or origin of these transferred animals was not detailed in the questionnaire.

4.3.3. CoproELISA results

In total, 5 out of 41 zoo enclosures (12.2%, 95%CI: 2.2-22.2) tested positive for *Echinococcus* spp. coproantigen in pooled faeces (Fig. 4-1). Each positive enclosure was located in a different zoo, meaning that overall, 5 out of 22 participating zoos (22.7%, 95%CI: 5.2-40.2) had a species enclosure testing positive for *Echinococcus* spp. coproantigen. All zoos returning a positive result were located in England.

Table 4-2. Summary of species, number, sex and age of canids and hyaenids at UK zoos participating in a survey of Echinococcosis UK zoo carnivores. Two zoos submitted faecal samples but did not submit completed questionnaires, therefore their data is classed as missing (Miss).

Species name	Common name	Total	> 6 months		Pups
			Male	Female	
Lycaon pictus	African Hunting Dog	3	3	0	0
Otocyon megalotis	Bat-eared Fox	2	1	1	0
Vulpes zerda	Fennec Fox	1	0	1	0
Canis lupus lupus	European Grey Wolf	16	6	10	0
Chrysocyon brachyurus	Maned Wolf	Miss	Miss	Miss	Miss
Canis lupus lupus	European Grey Wolf	3	2	1	0
Vulpes vulpes	Red Fox	2	0	2	0
Canis lupus signatus	Iberian Wolf	3	2	1	0
Vulpes vulpes	Red Fox	5	4	1	0
Lycaon pictus	African Hunting Dog	3	1	2	0
Speothos venaticus	Bush Dog	2	1	1	0
Canis lupus lupus	European Grey Wolf	2	1	1	0
Vulpes zerda	Fennec Fox	2	1	1	0
Crocuta crocuta	Spotted Hyena	3	1	2	0
Canis lupus lupus	European Grey Wolf	5	5	0	0
Vulpes lagopus	Arctic Fox	Miss	Miss	Miss	Miss
Canis lupus lupus	European Grey Wolf	Miss	Miss	Miss	Miss
Vulpes vulpes	Red Fox	Miss	Miss	Miss	Miss
Canis lupus lupus	European Grey Wolf	7	2	5	0
Chrysocyon brachyurus	Maned Wolf	2	1	1	0
Canis lupus lupus	European Grey Wolf	3	3	0	0
Canis lupus lupus	European Grey Wolf	3	2	1	0
Canis lupus signatus	Iberian Wolf	3	3	0	0
Vulpes zerda	Fennec Fox	2	1	1	0
Lycaon pictus	African Hunting Dog	8	8	0	0
Canis mesomelas	Black-backed Jackal	3	2	1	0
Vulpes corsac	Corsac Fox	10	4	6	0
Proteles cristata	Eastern Aardwolf	2	1	1	0
Chrysocyon brachyurus	Maned Wolf	1	1	0	0
Canis lupus lupus	European Grey Wolf	5	3	2	0
Vulpes vulpes	Red Fox	7	3	4	0
Canis lupus lupus	European Grey Wolf	2	1	1	0
Canis lupus signatus	Iberian Wolf	2	0	2	0
Speothos venaticus	Bush Dog	11	4	2	5
Otocyon megalotis	Bat-eared Fox	1	1	0	0
Chrysocyon brachyurus	Maned Wolf	5	2	3	0
Vulpes corsac	Corsac Fox	1	1	0	0
Canis lupus lupus	European Grey Wolf	3	1	2	0
Vulpes vulpes	Red Fox	3	3	0	0
Lycaon pictus	African Hunting Dog	7	0	7	0
Cuon alpinus	Dhole	19	5	14	0
	Total	**162**	**80**	**77**	**5**

One positive control sample from a domestic dog in Kyrgyzstan, testing positive for *Echinococcus* spp. coproantigen in voided faeces in a previous study (M. Rogan, personal communication), returned a mean OD below the cut-off in this study despite repeated assays. A second positive control from a known *Echinococcus* spp. positive domestic dog confirmed by purgation in the same study returned a mean OD above the cut-off threshold. A summary of relevant coproantigen sample data is shown in Table 4-3.

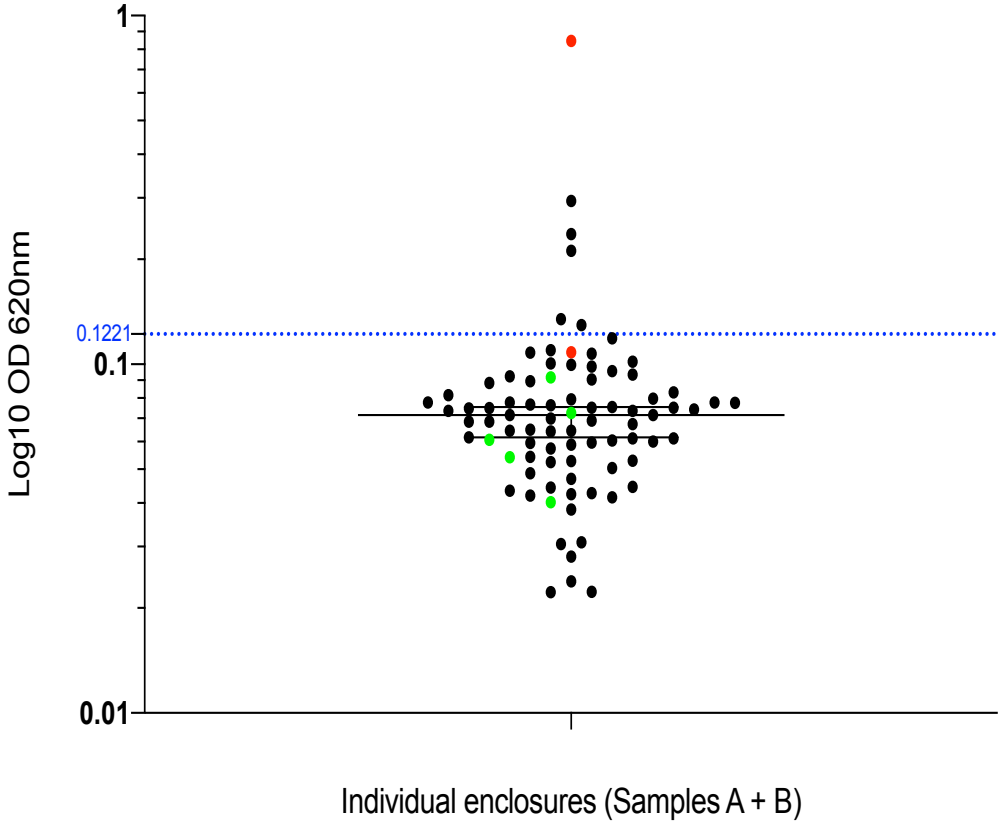

Figure 4-1. Echinococcus spp. coproELISA results for pooled zoo canid and hyaenid faecal samples from a survey of 41 UK zoos. Two pooled samples (A and B) for each species enclosure were tested. Mean OD 620nm readings are shown as a log scale with ● = positive control and ● = negative control. The cut-off value for test positivity is shown as 0.1221 OD

Table 4-3. Summary of genus specific Echinococcus coproELISA positive samples and controls from a study of wild canids and hyaenids in UK zoos.

Species	Common name	Mean OD 620nm
Lycaon pictus	African hunting dog	0.1348
Canis lupus lupus	European grey wolf	0.2117
Vulpes lagopus	Arctic fox	0.2365
Canis mesomelas	Black-backed jackal	0.2940
Canis lupus signatus	Iberian wolf	0.1295
Canis familiaris	Domestic dog	0.1081[a]
Canis familiaris	Domestic dog	0.8463[a]
Canis familiaris	Domestic dog	0.0917[b]
Canis familiaris	Domestic dog	0.0540[b]
Canis familiaris	Domestic dog	0.0600[b]
Canis familiaris	Domestic dog	0.0400[b]
Canis familiaris	Domestic dog	0.0725[b]

The cut-off value for a positive result was OD 0.1221 (620nm). [a] positive control, [b] negative control. All samples were assayed in triplicate and a mean OD value is given. All samples represent pooled voided faeces from a single animal enclosure.

4.3.4. CoproPCR results

The genus-specific multiplex coproPCR to identify *Taenia* spp. and *Echinococcus* spp., the *Echinococcus* s.l. coproPCR and the *E. granulosus* G1 coproPCR did not identify any positive samples, despite repeated assays and efforts to improve test specificity and sensitivity.

One pooled faeces sample from an enclosure of European grey wolves (*Canis lupus lupus*) tested positive for *E. equinus* by amplification and visualisation of a 299 bp DNA fragment within the NAHD dehydrogenase subunit (ND2) of the mitochondrial gene. A representative agarose gel image showing this result is shown in Appendix II-e.

4.3.5. Comparison of coproELISA and coproPCR

Pooled faecal samples from 5 enclosures located at 5 different zoo premises in England, each housing a different captive canid species tested positive for *Echinococcus* spp. coproantigen. In contrast, only one zoo had an enclosure testing

positive for *E. equinus* coproDNA. It was not possible to undertake an accurate statistical comparison of agreement between the tests, however the sole coproDNA positive sample found did not test positive for coproantigen, nor was coproDNA identified in any samples that had tested positive on coproELISA. This would suggest little agreement between the coprodiagnostic tests in this study. No significant associations were identified between species (p=0.216), being male (p=0.268) or female (p=0.855) and a positive coproELISA result.

4.3.6. Diet and feeding schedules

Overall, 20 out of 22 zoos provided details on diet and feeding schedules, although complete information was not entered for some cases. Frequency of feeding and whether feeding was communal or separate within the enclosure varied between species groups and zoo premises. The majority of enclosures, 25 out of 36 (69.4%, 95%CI: 54.4-84.5), were fed daily, with the remaining 11 out of 36 (30.6%, 95%CI: 15.5-45.6) incorporating 'fasted' days to replicate patterns of food availability in the wild. Such 'fasted' days were largely a part of feeding schedules for the larger canids, including wolf species (n=9), Spotted hyena (n=1), Dhole (n=1) and African wild dogs (n=1).

Almost all enclosures, 30 out of 32 (94.1%, 95%CI: 86.2-100), housing more than one animal were fed communally. Two enclosures at different zoos, one housing Maned wolves and another European grey wolves, fed animals separately due to specific dietary needs of individual animals within the group.

Of 22 zoos responding, 14 (63.6%, 95%CI: 43.5-83.7) indicated that they were licensed premises for the collection of animal by-products, 4 (18.2%, 95%CI: 2.1-34.3) indicated they were not. However, at the time of writing, all participating premises remained listed in the FSA approved list of ABP collection centres (APHA, 2018a).

Zoos were asked to detail the type of diet fed to individual species groups. Broad questionnaire categories included raw meat (flesh) or viscera (offal i.e. liver and/or lungs), further divided into species of origin (sheep, cattle, pig, horse, donkey, goat, poultry, rabbit, rodent) and whether sourced from fallen stock or from a butcher

and/or abattoir. Further categories included cooked meat or viscera (from any source), specialist or commercial, catering waste, fish or other (with an open text box for comment for any other category not included).

Table 4-4. Summary of types of diet fed to captive canids and hyaenids based on questionnaire data in a survey of 20 UK zoos accommodating 37 single-species enclosures. Counts are of zoos with at least one enclosure feeding a category of food and overall number of enclosures in all participating zoos, percentage of total and 95% Confidence Interval (95%CI).

Diet type	Species	Zoo			Enclosure		
		N	%	95% CI	N	%	95%CI
Fallen stock (Meat)	Cattle/Sheep	7	35.0	14.1-55.9	9	24.3	10.5-38.1
	Other mammals	7	35.0	14.1-55.9	10	27.0	12.7-41.3
	Poultry	6	30.0	9.9-50.1	10	27.0	12.7-41.3
Fallen Stock (Viscera)	Cattle/Sheep	6	30.0	9.9-50.1	7	18.9	6.3-31.5
	Other mammals	3	15.0	0.0-30.6	4	10.8	0.8-20.8
	Poultry	2	10.0	0.0-14.6	3	8.1	0.0-16.9
Abattoir/ Butcher (Meat)	Cattle/Sheep	9	45.0	23.2-66.8	16	43.2	27.3-59.2
	Other mammals	12	60.0	38.5-81.5	21	56.8	40.8-72.7
	Poultry	12	60.0	38.5-81.5	19	51.4	35.2-67.5
Abattoir/ Butcher (Viscera)	Cattle/Sheep	3	15.0	0.0-30.6	3	8.1	0.0-16.9
	Other mammals	1	5.0	0.0-23.1	1	2.7	0.0-7.9
	Poultry	2	10.0	0.0-14.6	2	5.4	0.0-12.7
Cooked (Meat)	Cattle/Sheep	1	5.0	0.0-14.6	3	8.1	0.0-16.9
	Other mammals	2	10.0	0.0-23.1	4	10.8	0.8-20.8
	Poultry	2	10.0	0.0-23.1	5	13.5	2.5-24.5
Other	Commercial	10	50.0	28.1-71.9	11	29.7	15.0-44.5
	Rabbits/rodents	16	80.0	62.5-97.5	20	54.1	38.0-70.1
	Catering waste	1	5.0	0.0-14.6	1	2.7	0.0-7.9
	Fish	8	40.0	18.5-61.5	12	32.4	17.3-47.5
	Fruit/veg/insect/egg	6	30.0	9.9-50.1	9	24.3	10.5-38.1

Overall, 20 out of 22 zoos provided details on diets fed to their canid and hyaenid collections. Zoos more frequently sourced raw meat and viscera from abattoirs or butchers than from fallen stock. Half of participating zoos, (10/20, 50%, 95%CI: 28.1-78.9) representing 14 out of 37 enclosures (37.8%, 95%CI: 22.2-53.5) fed red raw meat or viscera from fallen stock; 17/20 (85%, 95%CI: 69.4-100) zoos, representing 29 out of 37 enclosures (78.4%, 95%CI: 65.1-91.6) fed raw meat or viscera from abattoirs and/or butchers; 16 out of 20 zoos (80%, 95%CI: 62.5-97.5), representing 20 out of 37 enclosures (54.1%, 95%CI: 38.0-70.1) fed rabbits or rodents and 10 out of 20 zoos (50%, 95%CI: 28.1-78.9), representing 11 out of 37 enclosures (29.7%,

95%CI: 15.0-44.5) fed commercial or specialist proprietary food. Further information on types of diet fed by zoo and enclosures is reported in Table 4-4.

A further classification based on putative dietary risk of *Echinococcus* spp. infection was included. Of participating zoos, 7 out of 20 (35%, 95%CI: 14.1-55.9), representing 9 out of 37 enclosures (24.3%, 95%CI: 10.5-38.1) fed raw meat and viscera from sheep and cattle, classed as very high risk material for *E. granulosus* transmission to canid hosts (Buishi et al., 2006; Carmona et al., 1998; P. L. Moro et al., 1999). When including the feeding of raw material from other known viable intermediate hosts of *Echinococcus* spp., classed overall as high risk, this figure increased to 18 out of 20 (90%, 95%CI: 76.9-100), representing 31 out of 37 enclosures (83.8%, 95%CI: 71.9-91.7). This increase was largely due to the common feeding of raw meat and viscera of equine origin with 16 out 20 zoos (80%, 95%CI: 62.5-97.5) doing so to 30 out of 37 enclosures (81.1%, 95%CI: 68.5-93.7). Due to specific dietary needs, some species' enclosures were fed solely foodstuffs considered low risk for *Echinococcus* spp. infection e.g. commercial specialist food, poultry, insects, vegetables, fruit and fish; 6 out of 20 zoos (30%, 95%CI: 9.9-50.1) housed 6 out of 37 enclosures (16.2%, 95%CI: 4.3-28.1) with species solely fed these low risk foods. A summary of risk category of food fed to species enclosures is show in Table 4-5.

All enclosures testing positive on coproELISA were being fed material considered high risk for *Echinococcus spp.* transmission, with one being fed very high-risk material; no enclosures testing positive were feeding exclusively material considered a low risk for transmission. However, no significant associations were found between a coproELISA positive result and an enclosure being fed very high-risk material (p=1.000), high risk material (p=1.000) or low risk material (p=1.000).

Differences in the diets selected for the same species in different zoos suggested that there were no standard dietary recommendations for all species. For example, out of four enclosures of red fox included in the study, one was fed a very high-risk diet and another a very low-risk diet for *E. granulosus* transmission. One zoo reported feeding capybara carcases to its Bush Dog enclosure, although the origin of these carcases was not described.

Table 4-5. Numbers of enclosures by species feeding diets categorized by risk of infection with Echinococcus spp., including risk of E. equinus (meat or viscera from horses and donkeys) and very high risk for E. granulosus (viscera from sheep and cattle).

Species	Common name	Number of enclosures	*E. granulosus* very high risk	*Echinococcus spp.*		*E. equinus* risk
				risk	low risk	
Lycaon pictus	African Hunting Dog	4	1	4	0	4
Otocyon megalotis	Bat-eared Fox	2	0	1	1	1
Canis mesomelas	Black-backed Jackal	1	0	1	0	1
Speothos venaticus	Bush Dog	2	0	2	0	2
Vulpes corsac	Corsac Fox	2	0	1	1	1
Cuon alpinus	Dhole	1	1	1	0	1
Proteles cristata	Eastern Aardwolf	1	0	0	1	0
Canis lupus lupus	European Grey Wolf	10	4	10	0	10
Vulpes zerda	Fennec Fox	3	0	2	1	1
Canis lupus signatus	Iberian Wolf	3	2	3	0	3
Chrysocyon brachyurus	Maned Wolf	3	0	2	1	2
Vulpes vulpes	Red Fox	4	1	3	1	3
Crocuta crocuta	Spotted Hyena	1	0	1	0	1
	Total	37	9	31	6	30

4.3.7. Storage and disposal of food

Respondents were asked about the storage methods of foods fed to the zoo canid and hyaenid collections. Freezer storage was the most common method of food storage, used by 17 out of 20 zoos (85%, 95%CI: 69.4-100), representing 34 out of 37 enclosures (91.9%, 95%CI: 83.1-100). Refrigeration was used by 16 out of 20 zoos (80%, 95%CI: 62.5-97.5), representing 31 out of 37 enclosures (83.8%, 95%CI: 71.9-95.7); 2 out of 20 zoos (10%, 95%CI: 0.0-23.9), representing 3 out of 37 enclosures (8.1%, 95%CI: 0.0-16.9) stored items at room temperature.

Participating zoos were asked to give details on the method of disposal of food waste from captive canid and hyaenid enclosures. The majority of zoos, 18 out of 20 (90%, 95%CI: 76.9-100), representing 32 out of 37 enclosures (86.5%, 95%CI: 75.5-97.5) , disposed waste food via an incinerator off-site; no zoos had an on-site incinerator for this purpose. One zoo (5%, 95%CI: 0.0-14.6%) with a single enclosure (2.7%, 95%CI: 0.0-7.9) used a rendering plant and one zoo (5%, 95%CI: 0.0-14.6%) with four enclosures (10.8%, 95%CI: 0.8-20.8) used a waste disposal company.

4.3.8. Parasite control and prevention

Participating zoos were asked to detail whether they regularly wormed their canid and hyaenid collections and if so, the type(s) of wormer used and the frequency of administration.

Over half of participating zoos 12 out of 20 (60%, 95%CI: 38.5-81.5) reported regularly worming their canid and hyaenid collections, although this did not always represent all enclosures within a given zoo. Participating zoos reported administering regular worming in 19 out of 37 enclosures (51.4%, 95%CI: 35.2-67.5).

Out of 20 zoos returning questionnaire data, 16 responded with complete information about worming protocols used in their collections, three zoos gave information on some enclosures and one did not provide any information on worming. In total, worming information, complete or incomplete, was available for 30 out of 37 enclosures in the study.

Five wormer brands were used by participating zoos. All were combination products marketed for the treatment of common gastrointestinal parasites of domestic dogs and cats. Four of the wormer brands contained praziquantel (in combination with either milbemycin oxime or febantel and pyrantel embonate) and one contained fenbendazole as the sole component. No participants were using a product that solely contained praziquantel. Two of the wormers were of the legal category POM-V, to be supplied by a veterinary surgeon or under prescription and three were classified as NFA-VPS, to be supplied by a vet, pharmacist or specially qualified person (SQP) without prescription. The five wormers, licensed for use in domestic dogs were used under the cascade system (Veterinary Medicines Directorate, 2015).

There was substantial variation in the dose and frequency of wormer administration. Half of participating zoos 10 out of 20 (50%, 95%CI: 28-1-71.9) were administering a wormer containing praziquantel to at least one of their enclosures. However, this only represented 13 out of 30 enclosures (43.3%, 95%CI: 25.6-61.1), for which worming data were provided. Wormers containing fenbendazole as a combined roundworm and tapeworm treatment were used by 12 out of 20 zoos (60%, 95%CI: 38.5-81.5), representing 19 out of 30 enclosures (63.3%, 95%CI: 46.1-80.6).

In the absence of standardised worming protocols for the species in the study, and based on the general advice to extrapolate from recommended protocols for domestic dogs (AZA, 2012a), the study considered worming four times per year (or product instructions issued to that effect), as a baseline minimum for routine worming, as advised by ESCCAP for non-risk assessed dogs (ESCCAP, 2017). According to this classification, 4 out of 17 zoos (23.5%, 95%CI: 3.4-43.7) , representing 7 out of 27 enclosures (25.9%, 95%CI: 9.4-42.5) were worming according to this minimum protocol. Moreover, only 2 out of 17 zoos (11.8%, 95%CI: 0.0-27.1) representing 4 out of 27 enclosures (23.5%, 95%CI: 3.4-43.7) were administering a praziquantel wormer at this minimum protocol. No participating zoos were using the risk-based protocol of 6-weekly praziquantel worming recommended by ESCCAP for prevention of *E. granulosus* egg shedding in species fed potentially infective raw meat and viscera (ESCCAP, 2017).

Out of the six enclosures testing positive for *Echinococcus spp*. on coproELISA or coproPCR, two were using a wormer containing praziquantel, although both were doing so below the recommended minimum 3-monthly frequency.

A larger proportion of zoos administered wormers dependent on the outcome of faecal parasite testing, compared with those that administered regular prophylactic testing. Out of the 17 zoos that provided information about worming dose and frequency, 10 (58.8%, 95%CI: 35.4-82.2) administered wormer prophylactically to 17 out of 27 enclosures (63.0%, 95%CI: 44.7-81.2). Laboratory testing for the presence of parasites in faeces was undertaken by 17 out of 20 zoos (85%, 95%CI: 69.4-100) in 24 out of 37 enclosures (64.9%, 95%CI: 49.5-80.2). Table 4-6 provides a summary of the frequency of prophylactic worming and laboratory testing of faecal parasites.

Table 4-6. Summary of worming frequency and faecal testing for parasites of canids and hyaenids in 17 UK zoos for 27 enclosures (worming) and 24 enclosures (laboratory testing).

Frequency	Zoo			Enclosure		
	Number	%	95% CI	Number	%	95%CI
Worming		(n=17)			(n=27)	
Once yearly	4	23.5	3.4-43.7	5	18.5	3.9-33.2
Every 6 months	2	11.8	0.0-27.1	4	14.8	1.4-28.2
Every 3 months	4	23.5	3.4-43.7	7	25.9	9.4-42.5
As required by testing	7	41.2	17.8-64.6	11	40.7	22.2-59.3
Faeces laboratory testing		(n=17)			(n=24)	
Once yearly	3	17.6	0.0-35.8	5	20.8	4.6-37.1
Every 6 months`	11	64.7	42.0-87.4	15	62.5	43.1-81.9
Every 3 months	2	11.8	0.0-27.1	3	12.5	0.0-25.7
If parasites seen in faeces	1	5.9	0.0-17.1	1	4.2	0.0-12.2

Zoos using faecal testing as part of parasite control were asked to detail the type of laboratory used for this; 7 out of 16 zoos that provided this information, (43.8%, 95%CI: 19.4-68.1) used on-site laboratory facilities for the testing of faeces from 8/24 enclosures (34.8%, 95%CI: 15.3-54.2); 7 out of 16 zoos (43.8%, 95%CI: 19.4-68.1) used external laboratory services for faecal testing from 9 out of 23 enclosures (39.1%, 95%CI: 19.2-59.1) and 5 out of 16 zoos (31.3%, 95%CI: 8.5-54.0) used testing services at a local veterinary clinic from 7 out of 23 enclosures (30.4%, 95%CI: 11.6-39.2).

4.3.9. Enclosure flooring type, faeces collection and disposal

Participating zoos were asked to give information on the enclosure flooring type. More than one answer could be entered where applicable. Results suggest that canid and hyaenid enclosures have multiple flooring types and substrates and typically more than one within a given enclosure; out of 37 enclosures, 25 (67.6%, 95%CI: 52.5-82.7) included areas of solid flooring, 27 (73.0%, 95%CI: 58.7-87.3) included areas of soil cover, 26 (70.3%, 95%CI: 55.5-85.0) included areas of grass cover and 12 (32.4%, 95%CI: 17.3-47.5) added an additional substrate, such as sand, to solid floor areas.

Participating zoos were asked whether regular faeces collection was an integral part of enclosure management and if so, how faeces were then disposed. All zoos providing an answer to this question (n=18) reported routinely collecting faeces as part of enclosure management. Further details on methods of faeces disposal are presented in Table 4-7.

Table 4-7. Summary of faeces disposal methods among 18 UK zoos routinely collecting and disposing of faeces from 33 enclosures of canid and hyaenid species.

	Zoo			Enclosure		
Faeces disposal method	Number	%	95% CI	Number	%	95%CI
Muck heap	5	27.8	7.1-48.5	8	24.2	9.6-38.8
Refuse bin	3	16.7	0.0-33.9	3	9.1	0.0-18.9
Incinerator	5	27.8	7.1-48.5	7	21.2	7.3-35.2
Rendering plant	1	5.6	0.0-16.1	1	3	0.0-8.9
Farmer's fields	3	16.7	0.0-33.9	9	27.3	12.1-42.5
Waste management comp.	4	22.2	3.0-41.4	5	15.2	2.9-27.4

Overall, 8 out of 18 zoos (44.4%, 95%CI: 21.5-67.4), representing 17 out of 33 enclosures (51.5%, 95%CI: 34.5-68.6) disposed of faeces in ways that could potentially be spread onto agricultural and/or horticultural land i.e. to muck heaps and farmer's fields. A summary table of associations between coprodiagnostic test outcomes and questionnaire variables is shown in Appendix II-e.

4.4. Discussion

4.4.1. Study response

Echinococcus spp. infection was investigated in canid and hyaenid collections in 22 BIAZA member UK zoos, reflecting a participation rate of 51.2% among zoos invited to the study. It is likely that the high level of participation was positively influenced by the support of the BIAZA Research Committee for our work. This support and the willingness of zoos to collaborate, is an indication that the zoo community considers the risks of zoonotic disease in animal collections a research priority.

Due to the small population of zoos and wildlife parks holding canid and hyaenid collections, a random sampling strategy was not adopted. The aim of the study was to recruit as many zoos as possible to obtain samples from a wide variety of canid and hyaenid species. The convenience sampling design within BIAZA member collections has likely introduced selection bias in the study. BIAZA membership standards generally exceed the minimum species management requirements as set in the legislation (Draper, Browne, & Harris, 2013). As such, BIAZA members may undertake additional steps, under BIAZA policies and guidelines that reduce certain disease risks. By selecting study participants from this population, the level of *Echinococcus* spp. carriage in the wider UK zoo canid population may have been underestimated. Further studies would ideally include canids and hyaenids in all zoo animal collections in the UK, including non-BIAZA members and private canid collections not eligible for licensing under current zoo legislation (BIAZA, 2019; Great Britain., 1981).

4.4.2. CoproELISA and coproPCR results

Echinococcus spp. antigen was detected in the faeces of five canid species: African hunting dog, European grey wolf, Iberian wolf, Arctic fox and Black-backed jackal, located across five zoos in England. To the author's knowledge, this is the first report of *Echinococcus* spp. coproantigen positivity in these species in UK zoos. Although this identifies infection with *Echinococcus* at genus level only, the results suggest that

parasite transmission is occurring in multiple canid species in UK zoos, despite wide-ranging preventative health measures to mitigate infection and risk to public health.

Sylvatic lifecycles of *Echinococcus spp*. have been demonstrated for these species in several studies, with examples in Black backed jackals (Macpherson *et al.*, 1983; Wassermann *et al.*, 2015), African wild dogs (Hüttner and Romig, 2009; Kagendo *et al.*, 2014), Arctic fox (Stien *et al.*, 2010), Iberian wolf (Guerra *et al.*, 2013; Sobrino *et al.*, 2006) and European wolf (Shimalov & Shimalov, 2000; Sobrino *et al.*, 2006). To the author's knowledge, within these species in captivity in Europe, echinococcosis (*E. multilocularis*) has only been reported in a Eurasian wolf, a subspecies of the European grey wolf (Umhang *et al.*, 2016).

At species level, the study identified *E. equinus* coproDNA from pooled faeces in a European wolf enclosure in a zoo in England. To the author's knowledge, this is the first report of *Echinococcus* infection and the first finding of *E. equinus* demonstrated by coproPCR in a captive canid in the UK. Although not considered zoonotic, this finding has important implications for multi-species zoos housing both definitive and intermediate hosts for *E. equinus* in their collections. Two fatal cases of *E. equinus* infection have been reported in a Burchell's zebra and a red ruffed lemur in the UK. They were both in captive-bred animals that had not travelled outside of the country. It was suggested that their infection could have been introduced through forage and/or vegetables contaminated with *E. equinus* eggs then fed to the animals (Boufana *et al.*, 2012a). In the case of the Burchell's zebra, contamination of the zebra enclosure with the faeces of captive African wild dogs transported across the zebra compound to the muck storage area was also proposed (Boufana *et al.*, 2012). The finding of *E. equinus* in a captive canid raises the possibility of a transmission cycle occurring between definitive and intermediate hosts entirely within the zoo environment. The majority of enclosures in the study were fed ABP of equine origin, including all those testing positive on coproELISA and coproPCR. A definitive host infected by eating raw equine ABP containing viable hydatid cysts could contaminate its immediate environment with eggs passed in its faeces. Transfer of infective *Echinococcus* eggs from one site to another via shoes or vehicles acting as fomites or via wind, birds, beetles and flies has been suggested (Eckert & Deplazes, 2004). Foxes

were also known to have access to the zebra enclosure, though there is little data on the susceptibility of foxes to *E. equinus* nor have *Echinococcus spp*. isolates from UK foxes been genotyped (Boufana *et al.*, 2012). Red foxes (*Vulpes vulpes crucigera*) and arctic foxes (*Alopex lagopus*) have been successfully experimentally infected with protoscoleces derived from equine and ovine hosts from regions of England and Wales, although the resultant gravid adult parasites were putatively identified as *E. equinus* on gross morphology only (Cook, 1989).

The coproELISA and coproPCR tests used in the study are validated for coprodiagnostic use in domestic dogs' faeces species, not the captive canid species tested in this study. An assumption has been made that *Echinococcus* spp. coproantigen and coproDNA would be as detectable in captive canid and hyaenid species as they are in domestic dog faeces.

The host-pathogen ecology for many infectious diseases is poorly understood in many wild canid species, due to their endangered status and small populations in captivity (Grisham *et al.*, 1994). It is likely that transmission dynamics, infection pressures and host-pathogen relationships differ in captive environments. The reduced roaming range and physiological stress conditions encountered in a captive setting could alter the infection potential and shedding patterns of intestinal and other parasites (Geraghty, Mooney and Pike, 1981). Further study is needed to explore how these highly specialized environments alter transmission dynamics and risk to animal and human infection for pathogens such as *Echinococcus*.

4.4.3. Feeding practices

The study found that the majority of canid and hyaenid enclosures were fed daily and communally, with some enclosures, particularly wolf species, introducing 'fasted' days to mimic food availability in the wild. No significant associations were found between enclosure and the feeding of high-risk raw viscera. There was a trend towards the larger species of canid such as wolves, African wild dogs and Dhole, rather than smaller, more omnivorous species such as foxes or Bush dogs, receiving viscera as part of their diet, most likely through the practice of whole carcase feeding.

These three larger species are recognized as predominantly carnivorous and tend to prey collectively on ungulates many times their size. Given the increased likelihood of hydatid cysts occurring in visceral organs, it is possible that feeding behaviours place certain individuals at greater risk of infection. Research on how social behaviour and hierarchical structures influence feeding in many canid species is lacking (Dale *et al.*, 2017) and to the author's knowledge, there have been no studies investigating the potential influence of pack feeding behaviour on the risk of *Echinococcus* spp. transmission. Future collaboration with specialists in wild carnivore behaviour and ecology could identify species-related traits that my affect the risk of *Echinococcus* spp. infection and inform on resultant preventative measures. However, the overarching advice to mitigate transmission of many zoonotic *Echinococcus* spp. remains not to purposefully feed high-risk viscera to canids at all (Craig *et al.*, 2017).

The specialized nutritional needs of captive carnivores require careful development, and this was reflected in the varied and complex diets fed by different zoos and by species enclosure. Larger zoo collections may have a resident nutritionist who will carefully develop diets according to nutritional, physiological and behavioural needs of the species group.

Diet information was available for 4 out of 5 enclosures returning a positive *Echinococcus spp.* coproantigen result and for the single enclosure returning a positive *E. equinus* coproDNA result. All positive enclosures were feeding ABP of equine origin. In contrast, only one enclosure, positive on coproELISA, was feeding ABP of cattle origin. No enclosures testing positive were feeding ABP of sheep origin. Although a significant association between coprodiagnostic test positivity and diet type fed was not found, the trend suggests that coproantigen positive samples would more likely reflect an *E. equinus* than *E. granulosus* infection.

The questionnaire did not specifically ask whether Category 1 ABP from zoo animals was fed back to carnivores in the zoo. One zoo reported feeding of capybara carcases to an enclosure of Bush dogs, although the origin of the carcases was not described. It is presumed that they originated from fallen or surplus stock from zoo collections.

Bush dogs are the natural definitive host of *E. vogeli*, a zoonotic species, and capybara are a natural prey animal for this species. The role of capybara in the transmission cycle of *E. vogeli* in sylvatic cycles has been suggested (Manter *et al.*, 1972), but to date only identified in Agouti, another rodent prey species of Bush dog (do Carmo Pereira Soares *et al.*, 2014; Manter *et al.*, 1972). If feeding practices in UK zoos potentially replicate the sylvatic transmission cycle of *E. vogeli*, any definitive or intermediate hosts translocated from endemic countries into UK zoos should be considered a potential risk. The majority of zoos fed rabbits and rodents to at least one enclosure; this would typically be sourced from a specialist supplier and purchased as frozen carcases. Given the controlled indoor breeding conditions of such supplies, it is unlikely that commercially reared rodents would pose a transmission risk of *E. multilocularis*, even if sourced from a supplier outside the UK.

4.4.4. Parasite control and prevention

Over half (60%) of all participating zoos, representing 51% of all enclosures, reported deworming animals regularly as part of a preventative health programme. The study identified a variety of worming schedules used in zoos and species enclosures, including regular prophylactic worming and worming only upon a positive finding of parasites on faecal examination. The latter was a strategy employed by almost half (41.2%) of zoos in this study. Diagnosis of GI parasitic infections in zoo mammals typically involves standard qualitative and semi-quantitative coprological examination techniques, such as McMaster's method or Zinc Sulphate flotation/centrifugation (EAZA, 2014; Miller & Fowler, 2012). Although there is little detail in the literature of faecal testing protocols in use in zoos, at Johannesburg Zoological Gardens, carnivores testing positive for parasites on 3-monthly faecanalysis by egg flotation received a combination praziquantel/pyrantel wormer (Ramos, 2014). Worming only in response to positive faecanalysis results has a number of limitations. *Echinococcus spp*. and *Taenia spp*. eggs are morphologically identical and cannot be distinguished through microscopy alone (Craig *et al.*, 2003), limiting the value of diagnosis where identification of pathogenic and zoonotic parasite species is important. Intermittent shedding of eggs in faeces, pre-patent

infection, low worm burdens and the overdispersed nature of *Echinococcus* spp. in canids could lead to an underestimation of infection rates in individual and groups of animals (Torgerson & Deplazes, 2009) using standard coprological examination techniques. A negative test result may not be indicative of an animal's infectious status or its ability to shed an infectious agent in the future (DEFRA, 2012b). One zoo in the study indicated further testing only when parasites were grossly visible in the faeces. Although proglottids of *Echinococcus spp*. may occasionally be spontaneously discharged by definitive hosts in faeces (Eckert *et al*., 2002), the very small size of the parasite (2-6mm), combined with a likely low worm burden, would not make this method a reliable indicator of parasite carriage or egg shedding.

The composition of wormers used by participants largely fell into two groups: combination wormers containing praziquantel and milbemycin oxime or praziquantel, febantel and pyrantel embonate, licensed for treatment of common roundworms and tapeworms, including *Echinococcus spp*., or wormers containing fenbendazole alone, licensed for common roundworms and taeniid tapeworms, not including *Echinococcus spp*. Half of participating zoos returning information on worming practices were administering a wormer containing praziquantel, representing under half (43.3%) of enclosures in the study. The most commonly used wormers, by 60% of zoos in 63.3% of enclosures, contained fenbendazole as the sole anthelmintic agent in paste or granule formulations, rather than tablet form. All praziquantel-containing wormers used were only available in tablet form. It is possible that the granules or paste formulation of the fenbendazole-containing wormers was preferable to a tablet form for effectively delivering wormers in food to canid groups. A report of training of captive Dholes to accept tablet medication hidden in cubes of meat encountered challenges with animals rejecting medicated samples or spitting out the tablet from within pieces of meat (Williscroft, 2014). Administering wormers to zoo canids presents logistical challenges for keepers and vets, including achieving optimal weight-based dosing, ensuring all animals in a canid group receive a wormer, while causing minimal stress to the animals and ensuring safety of the operator.

Only two zoos overall were worming enclosures with praziquantel at the 3-monthly frequency that would reduce *Echinococcus spp*. infection and none at the recommended 6-weekly dosing that would prevent infection and egg shedding (ESCCAP, 2017). These findings suggest a very low level of preventative worming at participating zoos that would mitigate *Echinococcus spp*. infection risk in canids and hyaenids.

4.4.5. Disposal of faeces and ABP waste

This study found that all participating zoos reported regular removal and disposal of faeces from the canid or hyaenid enclosures. Hygiene measures within canid enclosures, including daily removal of faeces and uneaten food, reduced the incidence of gastrointestinal parasites and disease and levels of vermin (AZA, 2012a). This is in accordance with best practice management guidelines (AZA, 2012a; BIAZA, 2014; European Commission, 2015; DEFRA, 2012b).

Captive canids and hyaenids require natural substrates, such as soil or grass within enclosures to allow and encourage natural species-appropriate behaviours (AZA, 2012b). All enclosures in the study included a combination of surface types, with solid flooring and soil cover the most common. Alongside daily clearing of faeces from natural substrate flooring, the Association of Zoos and Aquaria (AZA) recommends daily cleaning and weekly disinfection of hard flooring areas, unless directed otherwise by a veterinarian (AZA, 2012b). Given the tenacity of cestode eggs in the environment, the concentration of animals in a defined enclosure and the difficulty in fully clearing natural substrate surfaces of all trace voided faeces, it is possible that enclosures over time, could concentrate a burden of *Echinococcus* eggs in the environment. Studies of microscopy and coproPCR of *Echinococcus* eggs in the soil of rural household gardens with domestic and wild canid access identified widespread *E. granulosus* egg contamination, presenting a potential for indirect transmission to humans (Shaikenov *et al.*, 2004). Such methods could also be used for soil and substrate sampling within the enclosure to determine the burden of environmental *Echinococcus* spp. egg contamination, although the success of such approaches as determinants of environmental parasitic egg contamination has been questioned

(Alvarez Rojas, Mathis, & Deplazes, 2018). The implications of such approaches would need to be carefully considered, including causing undue stress to animals in the enclosure, the time and resources required and any safety implications for the collector (AZA, 2012a; Fidgett, Plowman, & Whitehouse-Tedd, 2013). Zoo personnel responsible for daily clearing of enclosures should use appropriate personal protective equipment, such as disposable gloves, and employ regular hand washing to mitigate risks of infection from environmental faeces contamination (EAZA, 2014).

It is possible that the number and amount of faecal samples collected were not representative of the infection status within the enclosure. The number and amount of faecal samples were not determined relative to the number of animals in the enclosure, nor would it be known if samples collected came from different animals or whether coprophagia had taken place. For future studies, a more structured approach to sample collection, for example along a line transect of the enclosure, or using quadrants of known size could better represent parasite burden of animals in a known space (van Kesteren, 2015). However, such approaches would need to consider that many captive canid species tend to defecate around the perimeter of enclosures or will mark territory with site-specific defecation areas (AZA, 2012b).

The methods used by participating zoos for the disposal of faecal waste could lead to potential environmental contamination with *Echinococcus* eggs. Certain methods of faeces disposal i.e. onto a muck heap or spreading on farmer's fields could result in potentially infected faeces contaminating pasture grazed by livestock or used for cultivation of foods for human or animal consumption. This study found that almost half of all zoos and enclosures were disposing of canid and hyaenid faeces in ways that could potentially contaminate agricultural or horticultural land. To the author's knowledge, *Echinococcus* transmission risks to animals and humans associated with these methods of faeces disposal in zoos has not been explored. Many zoos sell manure from their collections as a commercial enterprise, either directly or through a distributor, often sold as 'zoo poo', or use it as compost on their own crop production or garden sites. Unprocessed manure is classed as category 2 ABP and may only be applied to land if it is deemed not to present a risk for the spread of any serious transmissible disease (DEFRA, 2011), however as the law classifies 'manure'

as excrement from farmed animals, the legislation relating to carnivore excrement as manure is unclear (European Commission, 2009).

4.5. Conclusions

This study of *Echinococcus spp*. in UK zoo canids and hyaenids has identified infection at the genus level in a number of species within different zoo collections. The finding of *E. equinus* coproDNA in a sample from a European grey wolf enclosure is of particular importance, given existing evidence of several fatal cases of cystic echinococcosis caused by *E. equinus* in intermediate hosts in UK zoos. DNA sequencing of this species-specific positive result would be a helpful confirmatory step and would allow a phylogenetic comparison with existing cases in UK zoo animals.

There is transmission potential of *Echinococcus* spp. through the feeding of high-risk raw ABP from fallen stock and abattoirs. This could better inform a risk-based approach to praziquantel wormer dosing and routine faecanalysis, which this study has shown to be largely unsuitable to mitigate *Echinococcus* infection risk. Assessments should cover both existing collections and imported animals, as under current legislation, there appears to be no provision for praziquantel worming of carnivore species imported from countries endemic for zoonotic *Echinococcus* species, including *E. multilocularis*, a notifiable disease in the UK.

The study has identified faeces disposal routes that could contaminate the environment, both within zoo premises and beyond to agricultural or horticultural land. The study has highlighted points where possible improvements could be made and where further research is needed to better understand the ecology of *Echinococcus* disease transmission in these captive animal groups. Communicating this information to stakeholders including BIAZA, zoo keepers and veterinarians is a key outcome of the thesis.

Chapter Five

Echinococcus spp. in UK farm dogs

5 *Echinococcus spp. in UK farm dogs*

5.1 Introduction

E. granulosus is transmitted principally through a domestic dog-sheep lifecycle and can infect other livestock species and humans as accidental dead-end hosts (Craig *et al.*, 2015). Farm dogs have been a focus of *E. granulosus* research in the UK, particularly in Wales, where prevalence of hydatid disease in humans has historically been at its highest (Buishi, Walters, *et al.*, 2005; Jones & Walters, 1992; Mastin *et al.*, 2011; Palmer *et al.*, 1996c). Farm dogs have also been the target of a large-scale public health intervention programme in Wales, to reduce the prevalence of echinococcosis in definitive and intermediate hosts (Buishi *et al.*, 2005; Craig & Larrieu, 2006; Palmer *et al.*, 1996c).

Working dogs on farms, particularly sheep farms, are trained to assist in the movement and protection of sheep flocks. In the UK, Collie breeds, particularly the Border Collie, are commonly, though not exclusively, bred and trained for working with sheep on both hill and lowland farms (ISDS, 2019). Dogs on farms may also be used for hunting, guarding or kept as pets.

5.1.1 Epidemiology of echinococcosis in farm dogs in the UK

5.1.1.1 Risk factors for Echinococcus infection in farm dogs

Dogs in rural settings or with access to open fields have higher reported risk of infection with *E. granulosus* than urban dogs (Buishi *et al.*, 2005; Acosta-Jamett *et al.*, 2010).

Within rural settings, farm dogs and sheepdogs have been identified at increased risk of *E. granulosus* infection (Buishi *et al.*, 2005; Moro & Schantz, 2009; Otero-Abad & Torgerson, 2013; Shaikenov *et al.*, 2003). Increased contact with livestock is understood to be a proxy for increased opportunity to scavenge fallen stock and access to the waste products of home slaughter (Moro et al., 1999; Otero-Abad & Torgerson, 2013).

Feeding of infected offal to dogs perpetuates transmission of *E. granulosus*. Farm dogs fed with hydatid-infected viscera are more likely to become infected (Buishi *et al*., 2006; Moro et al., 1999). Home slaughter in the UK is lawful under strict rules of animal welfare and food hygiene (FSA, 2013, 2018a; FSS, 2019). Practices that prevent access of dogs to livestock offal, such as not practicing home slaughter or ensuring proper disposal of carcase waste on farm, are associated with reduced risk of infection (Acosta-Jamett *et al.*, 2010).

The opportunity of dogs to roam freely has been reported frequently as a risk factor for *E. granulosus* infection (Buishi *et al.*, 2006; Huang *et al.*, 2008; Parada *et al.*, 1995). In Wales, roaming behaviour has been identified as a risk factor for *E. granulosus* infection in farm dogs (Buishi *et al.*, 2005; Mastin *et al.*, 2011).

A lack of knowledge of appropriate anthelmintic treatment has been identified as a risk factor for *E. granulosus* infection in rural dog populations (Acosta-Jamett *et al.*, 2014; Huang *et al.*, 2008; Parada *et al.*, 1995). In the UK, farm dogs that are wormed infrequently (<4 times per year) are more likely to test coproantigen positive for *Echinococcus* spp. (Buishi *et al.*, 2005). Differences in infection rates may be explained by roaming dogs being less likely to receive anthelmintic treatment than those kept as pets or guard dogs (Shaikenov *et al.*, 2003). Information about the adequacy of worming farm dogs in the UK is lacking, despite it being a population that has been shown to be at increased risk of both roaming behaviour and contact with livestock and fallen stock (Mastin *et al.*, 2011; Otero-Abad & Torgerson, 2013)

In a study of UK farm dogs, there was no association between whether the dog is working or non-working and *Echinococcus* spp. coproantigen positivity; an important finding that suggests pet dogs on-farm are also at risk of infection (Mastin *et al.*, 2011). Pet dogs on-farm may have greater access to the household than working farm dogs and increased human contact through petting. This presents an increased risk of human transmission (particularly in children), through carriage of infective *E. granulosus* eggs on their coat (Eckert *et al.*, 2001).

In rural and pastoral settings, an increased prevalence of infection has been reported in male dogs (Parada *et al.*, 1995), dogs less that 2 years of age (Buishi *et al.*, 2006; Buishi *et al.*, 2005) and in dogs over 5 years of age (Buishi *et al.*, 2005; Inangolet *et al*., 2010). To the author's knowledge, there is no census or report of how many working and non-working dogs live on farms in the UK.

Transmission of *E. granulosus* relies primarily on a cycle of infection between domestic dogs and livestock. Livestock infection occurs through the ingestion of viable eggs voided in faeces of infected dogs in the immediate home environment or on grazing pastures (Moro & Schantz, 2009; Otero-Abad & Torgerson, 2013). The farm environment presents a unique interface between humans, livestock, working and non-working dogs (belonging to the farm and the public via land access) and wildlife. There is very little information on practices such as collection and disposal of faeces of farm dogs and dogs belonging to the public and the potential associated risks. There is awareness and advice for the public to collect faeces, but little is known about whether this is a practice conducted on farms.

5.1.1.2 Echinococcus prevalence studies and control programmes in UK farm dogs

In the UK, most *Echinococcus* surveillance studies and control programmes in farm dogs have focused on known hotspots of endemicity in Wales (Buishi *et al.*, 2005; Jones & Walters, 1992; Mastin *et al.*, 2011; Palmer & Biffin, 1987). All studies have involved coproantigen techniques to diagnose genus specific *Echinococcus* spp. carriage in farm dog faeces. Such studies have been motivated by reported increases in autochthonous human case incidence in Powys and Brecknockshire regions of mid-Wales (Craig & Larrieu, 2002; Jones & Walters, 1992; Williams, 1976).

A number of surveys of *Echinococcus* spp. in farm dogs in mid-Wales reported dog-level prevalence of infection between 4.6-25.0% (Jones & Walters, 1992; Walters & Clarkson, 1980; Williams, 1976). Between 1983 and 1989, the South Powys Hydatid Control Scheme, run by the UK Ministry of Agriculture Fisheries and Food (MAFF), the State Veterinary Service and Agriculture Department of the Welsh Office, commenced 6-weekly supervised dosing of praziquantel anthelmintics of farm dogs

by MAFF staff. Concurrently, an education programme directed at sheep farmers and other dog owners, emphasised the importance of not feeding raw offal to dogs and the prevention of scavenging of sheep carcasses (Clarkson, 1978; Craig & Larrieu, 2006; Palmer *et al.*, 1996c). After 6 years, in 1989, funding for the scheme stopped and the praziquantel dosing programme was replaced by a health education programme, aimed at sheep farmers and school children, advising on health and safety, though farmers were encouraged to continue the 6-weekly worming of dogs at their own expense (Craig & Larrieu, 2006).

Following 5 years of active control measures, coproprevalence of *Echinococcus* spp. infection in the intervention area based on genus-specific coproELISA was 0% in farm dogs, compared with 2.4-9.2% in neighbouring non-control areas (Palmer et al., 1996c). However, further post-scheme surveys in 1996-2008 indicated an increased prevalence of transmission in farm dogs in the previous intervention areas, with reported prevalence of 6.3-8.8% at the dog level and 16.2-22% at the farm level (Buishi *et al.*, 2005; Lloyd *et al.*, 1998; Mastin *et al.*, 2011). The re-emergence of *Echinococcus* in farm dogs in Wales suggested that the premature ending of the dosing programme and its replacement with a health education programme did not control transmission in this area (Craig & Larrieu, 2006a).

Following the 2001 outbreak of Foot and Mouth Disease (FMD) in the UK, the resultant large-scale outdoor slaughter of sheep and cattle raised the concern of increased *E. granulosus* transmission from scavenging of carcasses lying in fields awaiting incineration. The risk to public health prompted a large-scale survey of echinococcosis in farm dogs in both FMD-affected and non-affected areas of Wales (Buishi *et al.*, 2005). The study in the Welsh counties of Powys and Gwent reported a coproantigen positive rate of 8.1% in farm dogs and 22% of farms surveyed but found no association between actions taken as a result of FMD control and coproantigen positivity in farm dogs. However, roaming and not worming adequately were identified as risk factors for a positive coproantigen result (Buishi *et al.*, 2005).

To date, coproantigen studies investigating *Echinococcus* spp. prevalence in UK farm dogs have assumed that positivity relates to infection with *E. granulosus*, rather than

E. equinus or other taeniid parasite. Only one study of canine echinococcosis in the UK has applied coproPCR techniques to confirm both *Echinococcus* genus and species carriage in farm dogs. A study of 20 farm dogs from farms in the Welsh counties of Powys and Wales using samples collected during previous coproantigen-based studies by Buishi *et al.* (2005) and Mastin *et al.* (2011) reported 15.0% of dogs had *E. equinus* DNA in their faeces and 85.0% had *E. granulosus* DNA (Boufana *et al*., 2015).

Online health education resources directed at sheep farmers are available via Public Health Wales (Public Health Wales, 2019), national farming organisations and farm assurance schemes. For example, the National Sheep Association (NSA) and Red Tractor Farm Assurance Standards Scheme have issued advice to member farmers regarding risk-based worming of dogs, advising that failing to worm farm dogs can affect productivity of livestock, cause offal and carcass rejections and put family at risk (Red Tractor Assurance, 2013; *NSA*,2019). As part of biosecurity and disease control measures within the Red Tractor Farm Assurance Standards Scheme, farmers are required to worm farm dogs regularly in accordance with manufacturer's recommendations and to record the product and date used (Red Tractor Assurance, 2013). There are no requirements on the anthelmintic coverage of the product. The assurance scheme also provides members with links to the National Animal Disease information Service (NADIS) (*NADIS,* 2019) for further advice on biosecurity risks, though the site currently does not include information on echinococcosis or prevention of hydatid disease in sheep flocks.

5.1.2 The National Sheep Association

This study was undertaken in collaboration with the NSA, an organisation representing the interests of sheep producers throughout the UK, with a membership of over 6,500 farms, from which participants were recruited.

The NSA has 11 member regions, 9 within the UK and two overseas (Appendix III-a). Policy work includes promotion of best practice in sheep health via the Healthy Flocks Programme, in association with the Sheep Veterinary Society (NSA, 2016). Through

online resources, sheep events, monthly Sheep Farmer magazine, the programme aims to improve sheep health, genetics and nutrition of sheep flocks.

The National Sheep Association informs member farmers about the risk of tapeworm parasites transmitted by dogs although this is mainly directed towards control of *C. ovis* (sheep measles) (NSA, 2019b). The NSA issues advice to member farmers to worm with praziquantel every 6 weeks and not to permit dogs onto pasture in the 48 hours following worming. In addition, they recommend preventing dogs having access to sheep carcasses and placing polite notices requesting dog walkers with dogs to regularly worm their dogs.

5.1.3 Study aims

There is a lack of up-to-date knowledge on prevalence of *Echinococcus* in farm dogs within and outside of known regions of high prevalence. The role that farm dogs and farm dog owner-associated risk factors play in the emergent and re-emergent picture of echinococcosis in the UK requires further urgent investigation.

The proposed study had three main aims. The first aim was to assess the proportion and location of NSA member farms in the UK testing positive for *Echinococcus* spp. infection at the farm level through the pooled faecal testing of all dogs on the farm.

The second aim was to investigate farm dog husbandry factors important to the transmission dynamics of *Echinococcus*, including specific diets, land access and scavenging behaviour, detailed worming practices and collection and disposal of faeces and analyse the resultant data for associations with coprological test positivity.

Thirdly, the study aimed to test the hypothesis that *E. granulosus* in farm dogs is not confined to historic areas of high prevalence in Wales by undertaking a freedom-from-disease analysis of the nine participating UK NSA member regions.

Overall, the study will contribute to the understanding of *Echinococcus* spp. distribution in the UK to better inform surveillance, public health information and future control efforts.

5.2 Methodology

The methodology for this study broadly follows the general questionnaire and faecal sampling approach and laboratory protocols for the canine cross-sectional studies described in Chapter 2. A number of modifications, specific to this study design and recruitment strategy, are described here.

5.2.1 Study design

This study was undertaken in collaboration with the NSA. Participants were recruited from UK members listed in June 2018 and the study unit was the individual farm. The study design was a prospective cross-sectional study to investigate *Echinococcus* spp. in the faeces of farm dogs, survey factors that could influence *Echinococcus* infection rate and demonstrate freedom from *Echinococcus* spp. carriage in NSA member farms within UK member regions. Ethical approval was granted by the University of Liverpool Veterinary Research Ethics Committee (VREC654) in March 2018 and sampling commenced in June 2018. Inclusion criteria for participants in the study were NSA members' farms with resident working or non-working dogs on-farm.

A study questionnaire and participant information sheet (Appendix III-c, III-d) were designed using Microsoft Word 2010 (Microsoft Corporation, USA). The questionnaire was divided into sections on dog numbers, husbandry, feeding, housing, exercising, worming and faeces disposal, following the general format as described in Chapter 2. Study-specific modifications to the general questionnaire included whether dogs were working or non-working, their access to land on the farm, whether the farmer had witnessed dogs on the property scavenging fallen stock and whether the farmer displayed worming safety notices for the general public accessing their farmland. The questionnaire format required mostly tick-box responses, with occasional open-ended questions where appropriate. The questionnaire was reviewed by a senior livestock researcher at the NSA and piloted by a panel of researchers at the University of Liverpool not involved in the study. Participants were also asked to collect freshly voided faecal samples from all dogs kept on farm using a sampling kit provided by the researcher. If more than two dogs

were kept on the farm, participants were advised to pool samples into the two pots provided, up to a maximum of 16 samples overall. The aim was to sample all dogs present on the farm, whether by individual or pooled samples.

Questionnaires and faecal samples were returned by First Class Royal Mail post in accordance with UN3373 Sample Transport Compliance Regulations (Department for Transport., 2012) A detailed description of the sampling kit and postal arrangements is described in Chapter 2.

Results of the coproELISA and coproPCR tests were reported to participants on request and any participant wishing to know test results relating to individual dogs were asked to include the name of the sole dog providing the sample on the sample pot. Participants were advised that if pooled samples were submitted, a generic positive or negative result would be reported.

At the time of sampling, the NSA listed 6529 members divided into 11 UK member regions including Scotland (n=854), Wales (n=1356), Northern Ireland (n=190) and six regions in England: Northern (n=1078), Central (n=439), Eastern (n=286), Marches (n=736), South East (n=696) and South West (n=835). Two member regions of EIRE/Europe and Overseas (outside Europe) were not included in the study. The proportions of membership within the regions broadly matched the recent geographic density maps of the sheep population and agricultural sheep holdings in GB (NI data density data not available) (Appendix III-b) (APHA, 2018b).

Sample size calculations for each region were based on estimating freedom of disease (assuming a perfect test) (Dohoo, 2010). The following formula was used to calculate the minimum sample number per region to estimate freedom of disease within the region using the farm as a study unit:

$$n = (1 - (\alpha)^{\frac{1}{D}})(N - \frac{D - 1}{2})$$

Where:

$$n = required\ sample\ size$$

$$\alpha = 1 - confidence\ level\ (0.05)$$

$$\mathrm{D} = estimated\ min.no.diseased\ farms\ per\ group \begin{pmatrix} region\ membership \\ x \\ min.expected \\ prevalence \end{pmatrix}$$

$$N = number\ of\ farms$$

To the author's knowledge, no prevalence studies of *Echinococcus* spp. in UK farm dogs have been conducted outside the known hotspot areas of mid-Wales, where studies have reported farm-level coproantigen prevalence of between 0.0-22.0% (Palmer *et al.*, 1996b; Buishi *et al.*, 2005; Mastin *et al.*, 2011) and dog-level coproantigen prevalence of 0.0-8.8% (Palmer *et al.*, 1996b; Lloyd, Walters and Craig, 1998; Buishi *et al.*, 2005; Mastin *et al.*, 2011). It was anticipated that farm and dog level *Echinococcus* spp. prevalence in other UK areas would be considerably lower. A minimum expected prevalence at farm level of 6.0% was estimated for this study, based on half of the average farm-level prevalence reported in the hot spot areas of Wales.

Sample size calculations indicated between 49 and 57 farms would need to be recruited per region to detect freedom of disease, depending on the number of members per region. Overall, the study aimed to recruit a total of 500 NSA member farms from the nine UK member regions. In order to allow for an expected low response rate and an unknown number of member farms keeping dogs, up to three rounds of recruitment, each randomly selecting the required number of farms per region was undertaken.

If insufficient farms were recruited from a region, one or more adjacent regions would be amalgamated, requiring a proportionally lower sample size to allow freedom of disease calculation within the larger combined areas.

Farms were recruited from an anonymised regional member list provided by the NSA. Random sampling from the anonymised member list was conducted using the Epitools epidemiological calculator for simple random sampling without replacement (Sergeant, 2018). Randomly selected farms were contacted directly by the NSA via

email or letter and asked to respond directly to the lead researcher with contact details if they wished to participate. Using this method, only farms willing to participate were invited to the study. During each recruitment round, the NSA also included a notice in the weekly email newsletter to all members informing them of recruitment taking place.

5.2.2 Statistical analysis

To describe proportions of farms engaging in farm dog husbandry and healthcare practices of interest, questionnaire data are presented as proportion and percentage with 95% confidence intervals (95%CI). A two-sided Fisher's exact test was used to investigate associations between coprological test positivity and *a-priori* risk factors for infection at the farm level, including geographical location, raw food feeding, scavenging behaviour, sub-optimal worming and faeces disposal, with significance set at $p<0.05$ (i.e. Type I error (α) = 0.05). For assessment of agreement between coproELISA and coproPCR dichotomous test results, a McNemar's test was initially used to establish that both tests classified approximately the same portion of dichotomous results as positive, with significance set at $p<0.05$. A Cohen's Kappa statistic was then used to measure the level of agreement between coproELISA and coproPCR G1 test results at the farm level. All statistical analyses were performed using Stata 14 (StataCorp, 2015). Analysis and graphical display of coproELISA data was performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA). Geolocation was completed with QGIS v2.18.14 using InFuse UK Data Service census administrative base maps (*Office for National Statistics (2011): 2001 Census aggregate data (Edition: May 2011). UK Data Service.*, 2011).

5.2.3 CoproELISA

Chapter 2 describes in detail the laboratory protocol of the coproELISA assay conducted in this study. In brief, pooled faeces samples received were frozen at -80˚C for minimum 1 week prior to further processing to destroy infective stages of cestode parasites (WHO/OIE, 2001; van Kesteren, 2015, Carabin *et al.*, 2005) and stored at -20C thereafter. Genus-specific *Echinococcus* spp. coproantigen in farm dog faeces

supernatants was identified using an internationally recognized protocol based on the methods of Allan *et al.* (1991) and Craig *et al.* (1995), further optimised at the University of Salford by van Kesteren *et al.* (2013).

A farm was classified as coproELISA positive if one or more single or pooled submitted farm dog faeces samples gave a mean coproELISA OD reading (to 3 significant figures) above the defined cut-off value of 0.2 OD at 620nm. This revised cut-off value specific for the farm dog study, was calculated via a panel of 21 known negative faecal samples (selected from those previously included in the Gaussian negative panel assay described in Chapter 2), run in triplicate using the same conditions and reagents. The cut-off value of 0.2 represents the mean of the replicate ODs plus 3 standard deviations. The calculation of this specific cut-off value was necessary due to a persistent higher OD value in all repeated runs of the farm dog coproELISA test, despite extensive troubleshooting of the protocol. The reason for this persistent assay-wide higher OD value compared to other HyData study assays is unknown. As such, the Gaussian cut-off value of 0.122 OD developed from the negative sample panel and used for the hound and zoo coproELISA studies was not suitable for the farm study. The revised cut-off value was able to reliably distinguish between positive and negative control samples.

Negative control samples were from pet dogs in England volunteered by colleagues of the researcher; dogs were fed solely non-raw proprietary dog food and were recently wormed with praziquantel. Positive control samples were from known *E. granulosus* G1 infected dogs in Kenya and Kyrgyzstan, confirmed by purgation in previous studies (van Kesteren *et al.*, 2013, M.Rogan, personal communication) or known negative faecal samples 'spiked' with *E. granulosus* G1 whole worm extract.

5.2.4 CoproPCR for *Echinococcus spp*., E. granulosus (G1) and E. equinus (G4)

Chapter 2 describes in detail the coproPCR protocols used in this study. In brief, coproDNA was extracted from farm dog faeces samples using the QIAmp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer instructions for large volumes of starting material. The presence of genus-specific *Taenia* spp. and

Echinococcus spp. coproDNA was investigated using the multiplex protocol described by Trachsel *et al.* (2007). The protocol by Abbasi *et al.* (2003) was used to identify *E. granulosus* coproDNA. Species-specific *E. granulosus* G1 and *E. equinus* coproDNA were investigated using protocols by Boufana *et al.* (2013) and Lett *et al.* (2018) respectively. PCR amplification products were identified from their size using gel electrophoresis and products were sequenced for further confirmation (Source Bioscience, UK).

5.3 Results

5.3.1 Study response and participation

At the time of study recruitment, there were 6470 registered UK members of the NSA. During the recruitment period of June 2018 until May 2019, three randomised recruitment rounds took place, each over a period of 3 months. In each round, between 49 and 57 farms were randomly selected per region, depending on the minimum sample number per region required to estimate freedom from disease. A total of 497 randomly selected anonymised member farms were invited to participate per round and a total 1491 farms over the duration of recruitment. A response was defined as a farm contacting the author to express interest in taking part. Participation was defined as a farm returning a completed questionnaire and farm dog faecal sample.

In Round 1, a total of 17 out of 497 members replied to the study, giving a response rate of 3.4% (95%CI 1.8-5.0). One respondent did so to inform that he no longer kept sheep or sheep dogs. Of the members who responded with interest in participation and were sent the sample kit, 15/16 returned the sample and completed questionnaire, giving a participation rate of 93.8% (95%CI 81.9-100) within the response group and 15 out of 497 (3.0%: 95%CI 1.5-4.5) within the round. From an advert placed in the NSA newsletter, a further 2 members volunteered participation following the newsletter advert.

In Round 2, there was a response rate of 19 out of 497 (3.8%, 95%CI: 2.1-5.5) and a participation rate of 11 out of 19 (57.9%, 95%CI: 35.7-80.1) farms within the response

group and 11 out of 497 (2.2%, 95%CI: 0.9-3.5) within the round. No volunteer members contacted the study to take part in Round 2.

In Round 3, there was a response rate of 31 out of 497 farms (6.2%, 95%CI: 4.1-8.4). Two members responded to inform that they no longer kept sheep or sheep dogs. With these two members excluded, the round gave a participation rate of 17 out of 29 (57.9%, 95%CI:35.7-80.1) within the interested group and 17 out of 497 (3.4%, 95%CI: 1.8-5.0) within the round. From an advert placed in the NSA newsletter, a further 3 members contacted the study to volunteer participation.

The total response rate for the study was 67 out of 1491 farms (4.5%, 95%CI: 3.4-5.5), respondent participation rate 47 out of 63 farms (74.6%, 95%CI: 63.9-85.4) and total participation rate 47 out of 1491 farms (3.2%, 95%CI: 2.3-4.0).

During the third and final round of recruitment, it was not possible to randomly select from a full list of remaining farms in Scotland and Wales. Due to a concurrent research survey in these regions, the NSA requested that members were not invited to participate in two research surveys at the time. To overcome this, a further round of randomised selection within the regions was undertaken, omitting any farms taking part in the other survey, but ensuring that the required number of farms as per the study design were selected for invitation.

Unfortunately, insufficient farms were recruited from each NSA member region to reach the minimum calculated sample numbers for calculation of freedom from disease in each region or the minimum combined regions.

5.3.2 Regional distribution of participants

In total, 47 NSA member farms participated in the study. The study found a significant association between a positive result on either coprological *Echinococcus* spp. test conducted and farm location in the NSA Wales member region ($p<0.05$) (Appendix III-e). The highest rate of participation of 6% (95%CI 2.4-9.5) was in the Marches region and the lowest rate of 1.2% (95%CI 0-2.8) in the South East region (Table 5-1).

Table 5-1. Summary of regional recruitment in a cross-sectional study of 1491 NSA member farms

NSA Region	Number of farms			% (95%CI)		
	NSA	invited	received	participation	Membership	of overall participants
Central	439	165	3	1.8 (0-3.9)	0.7 (0-1.5)	6.4 (0-13.6)
Eastern	286	162	3	1.9 (0-3.9)	1 (0-2.2)	6.4 (0-13.6)
Marches	736	168	10	6.0 (2.4-9.5)	1.4 (0.5-2.2)	21.3 (9.6-33.0)
N. Ireland	190	147	5	3.4 (0.5-6.3)	2.6 (0.4-4.9)	10.6 (1.8-19.5)
Northern	1078	171	7	4.1 (1.1-7.1)	0.6 (0.2-1.4)	14.9 (4.7-25.1)
Scotland	854	168	7	4.2 (1.1-7.2)	0.8 (0.2-1.4)	14.9 (4.7-25.1)
South East	696	168	2	1.2 (0-2.8)	0.3 (0-0.7)	4.3 (0-10.0)
South West	835	171	6	3.5 (0.8-6.3)	0.7 (0.1-1.3)	12.8 (3.2-22.3)
Wales	1356	171	4	2.3 (0.1-4.6)	0.3 (0-0.6)	8.5 (0.5-16.5)
Total	6470	1491	47	3.2 (2.3-4.0)	0.7 (0.5-0.9)	100

5.3.3 Dog populations on participating farms

A total of 158 dogs were reported on participating farms. The number of dogs at a farm premises ranged from 1 to 13, with a median number of 3 dogs (IQR 2-4) per farm. There was no statistically significant association between NSA member region and the number of dogs per farm (p=0.502). The questionnaire gathered data on the working status of the dogs, their sex and whether adult or puppy (i.e. less than 6 months old). The majority of dogs were used for working with sheep, with 110 out of 158 classed as working (69.6%, 95% CI: 62.4-76.8) and 48 out of 158 classed as non-working (29.6%, 95% CI: 23.2-37.6) (retired working or pet dogs). There was a greater number of female dogs than male dogs in both working and non-working groups. Only two participating farms reported having pups (less than 6 months of age) on farm. A summary of these data are shown in Table 5-2.

Table 5-2. Summary data on sex and working status of 156 farm dogs in a cross-sectional study of NSA member farms.

		Number	Percent	95% CI
Working	Male	47	29.0	22.0 - 36.0
	Female	61	37.7	30.2 - 45.1
	Pup (< 6 months)	2	1.2	0 .0 - 2.9
	Total	110	70.4	63.3 - 77.4
Non-working	Male	21	13.0	7.8 - 18.1
	Female	26	16.0	10.4 - 21.7
	Pup (< 6 months)	1	0.6	0.0 - 1.8
	Total	48	29.6	22.6 - 36.7

The Marches region had the highest proportion of working dogs with 25 out of 110 (22.7%, 95% CI: 14.9-30.6) and the Eastern region the lowest, with 3 out of 110 (2.7%, 95% CI: 0-5.8). Northern Ireland had the highest proportion of non-working dogs on participating farms, with 9 out of 48 (18.8%, 95% CI:10.2-31.9) and Wales the lowest with 1 out of 48 (2.1%, 95% CI: 0-6.1). A summary of the regional data is shown in Table 5-3. and Figure 5-1.

Table 5-3. Summary data on the working status of 158 farm dogs on 47 farms across 9 NSA member regions in a cross-sectional study of NSA member farms.

Region	Number of farms	Working dogs			Non-working dogs		
		Number	% total	95%CI	Number	% total	95% CI
Central	3	11	10	4.4 - 15.6	3	6.3	0.0 - 13.1
Eastern	3	3	2.7	0.0 - 5.8	2	4.2	0.0 - 9.8
Marches	10	25	22.7	14.9 - 30.6	8	16.7	6.1 - 27.2
Northern	5	13	11.8	5.8 - 17.9	8	16.7	6.1 - 27.2
N. Ireland	7	7	6.4	1.8 - 10.9	9	18.8	7.7 - 29.8
Scotland	7	11	10	4.4 - 15.6	8	16.7	6.1 - 27.2
South East	2	8	7.3	2.4 - 12.1	5	10.4	1.8 - 19.2
South West	6	20	18.2	11.0 - 25.4	4	8.3	0.5 - 6.1
Wales	4	12	10.9	5.1 - 16.7	1	2.1	0 .0 - 6.1
Total	**47**	**110**	**100**		**48**	**100.0**	

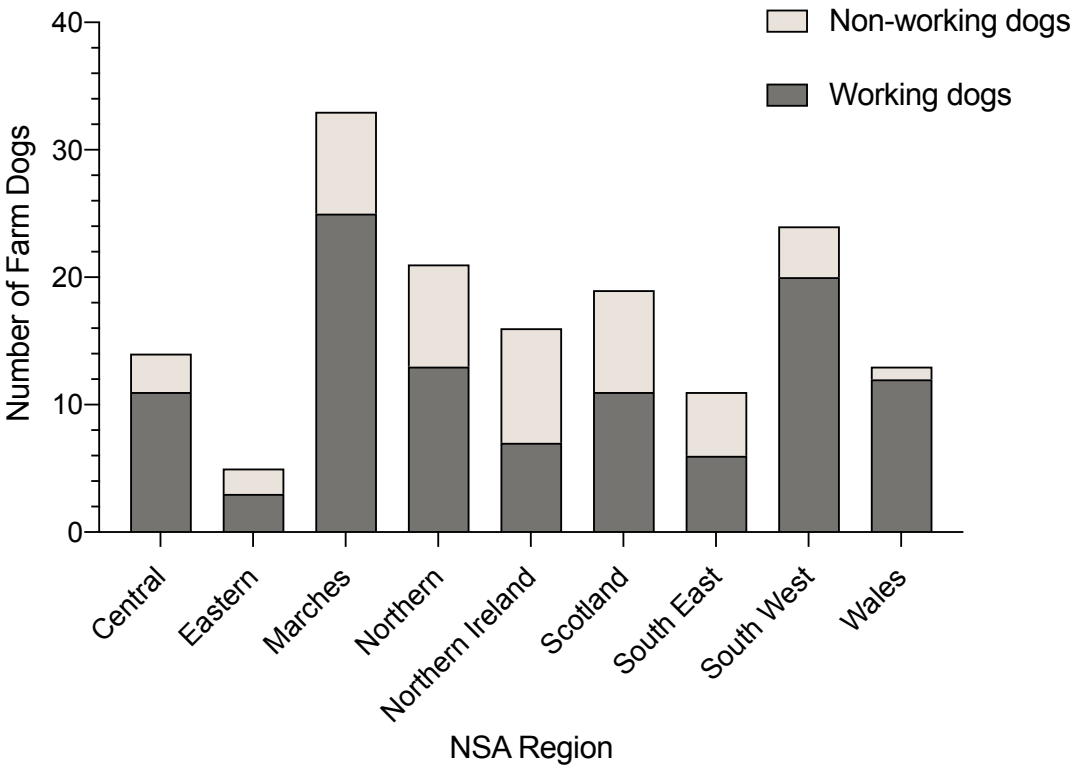

Figure 5-1. Number of working and non-working farm dogs at participating farms within UK NSA regions.

5.3.4 CoproELISA results

Of farms tested, 8 out of 46 17.4% (95%CI 6.4-28.3) kept at least one farm dog testing positive for *Echinococcus spp*. at the genus level. A total of 11 out of 86 faecal samples (12.8%, 95%CI: 5.7-19.8) tested positive for *Echinococcus* spp. antigen on coproELISA Figure 5-2. The samples represented dogs from 8 different farms (Table 5-4). In 3 out of 8 farms (37.5, 95%CI 4.0 -71.0), positive results were found on both samples, resulting in likely more than one dog being positive on that farm.

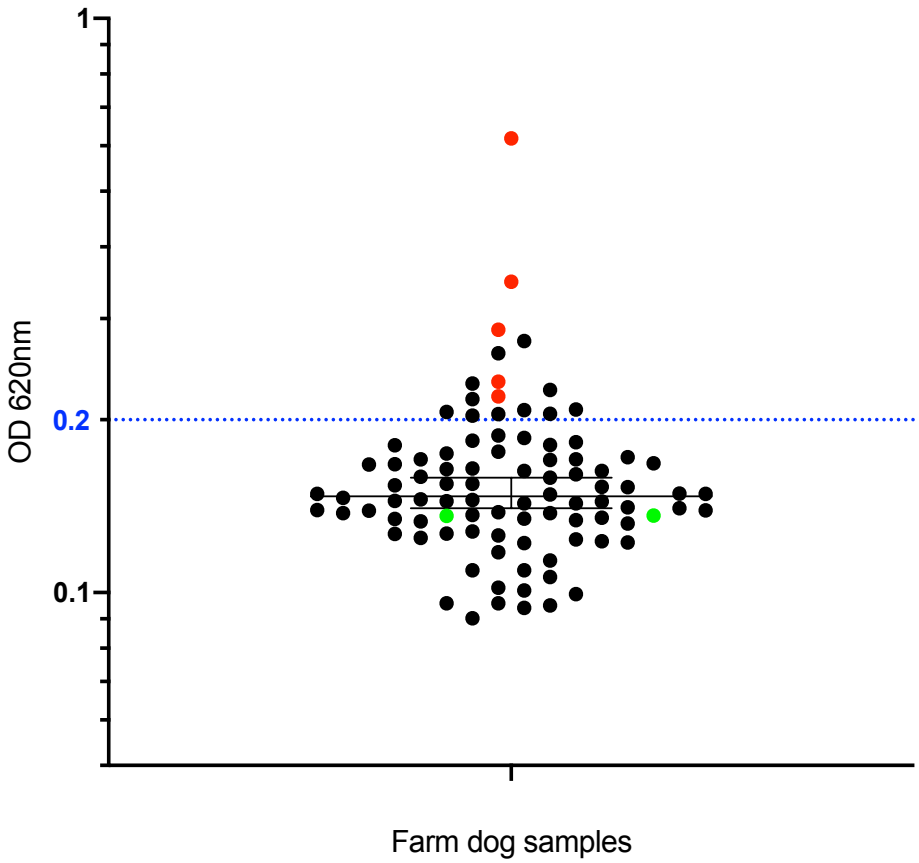

Figure 5-2. I. coproELISA results for farm dog faecal samples from NSA *member farms participating in a cross-sectional survey*. *OD 620nm readings are shown as a log scale with ● = positive control and ● = negative control. The cut-off value for test positivity is shown as 0.2 OD 620nm. Horizontal lines represent the mean OD and 95%CI.*

Of the 8 farms with dogs testing positive on coproELISA, 6 were in England, one in Scotland and one in Wales. Of the farms testing positive in England, three were located in the Northern NSA member region, one in the South East, one in the Eastern

and one in the Marches region. Approximate location of farms with dogs testing coproantigen are shown in Figure 5-3.

Table 5-4. Summary of genus-specific Echinococcus coproELISA positive samples and controls from a cross-sectional study of 47 NSA member farms.

Farm/control sample	Mean OD	NSA region	Country
13A	0.2084	South East	England
14A*	0.2047	Wales	Wales
14B*	0.2078	Wales	Wales
22A	0.2034	Northern	England
25B	0.2064	Scotland	Scotland
27A*	0.2049	Northern	England
27B*	0.261	Northern	England
40A	0.2314	Eastern	England
42A*	0.2173	Northern	England
42B*	0.2255	Northern	England
45A	0.2742	Marches	England
Pos 1	0.348	na	Kenya
Pos 2	0.287	na	Kenya
Pos 3	0.233	na	Kenya
Pos 4	0.2198	na	Kyrgyzstan
Pos 5a	0.6183	na	England
Neg 1	0.1361	na	England
Neg 2	0.1362	na	England

Individual farms are identified by a numerical code and pooled samples from the farm identified as A or B. * denotes both samples submitted from the same farm. aNegative faecal sample 'spiked' with *E. granulosus* whole worm extract to give a positive control sample. Cut-off value for a positive result was OD 0.2 at 620nm. Pos = positive control, Neg = negative control, na= not applicable. All samples were assayed in triplicate and a mean OD value is given. All samples represent single dogs.

5.3.5 CoproPCR results

Neither the genus-specific multiplex coproPCR to identify *Taenia* spp. and *Echinococcus* spp. or the *Echinococcus* s.l. coproPCR identified any positive samples, despite repeated assays and efforts to improve test specificity and sensitivity.

However, using the coproPCR for *E. granulosus* G1, a total of 5/46 (10.9%, 95%CI 1.9-19.9) farms were identified as having at least one dog testing positive on coproPCR. A representative agarose gel image showing this result is shown in Appendix III-e.

None of the samples tested positive for *E. equinus.* A total of 6 samples on the 5 farms tested positive on coproPCR specific for *E. granulosus* G1 genotype by amplification and visualisation of a 226bp DNA fragment within the NAHD dehydrogenase subunit (ND1) of the mitochondrial gene. In all cases, bands visualised on a gel were very feint but visible. Unfortunately, it was not possible to successfully isolate a viable DNA product from positive samples for sequencing as a confirmatory step. Two farms (farm ID 7 and 28) with dogs testing positive, were in the NSA Wales region (farm ID 7 with both submitted samples positive), one farm (ID 22) in the Northern region, England, one farm (ID 25) in Scotland and one farm (ID 44) in Northern Ireland.

5.3.6 Comparison of coproPCR and coproELISA test results

A total of 11 farms had at least one positive dog by either coproELISA and/or coproPCR assay. Two farms, one in Scotland and one in the Northern region, submitted samples testing positive on both assays (Figure 5-3). A summary table of coprodiagnostic test results at the farm level by NSA member region is shown in Table 5-5. In the absence of any samples positive for *E. equinus* in this study, or evidence of other *Echinococcus* species in the UK other than *E. granulosus* G1, an assumption was made that a positive result on genus-specific coproELISA suggested the presence of *E. granulosus* G1 in the sample. There was little evidence that the two tests found significantly different proportions of results positive (p=0.5271), suggesting it was possible to compare agreement between the tests. On comparison of coproELISA and coproPCR G1 results at the farm level there was 78.26% agreement (κ=0.1606, p=0.1346). A κ value between 0.01 and 0.2 suggests only slight agreement between the tests (Landis & Koch, 1977). A summary table of associations between coprodiagnostic test outcome and questionnaire variables is located in Appendix III-f.

Table 5-5. Summary of coproELISA and coproPCR G1 results at the farm level by NSA member region.

NSA region	Farm ID	CoproELISA	CoproPCR G1
Scotland	25	Yes	Yes
Northern	22	Yes	Yes
	27	Yes*	No
	42	Yes*	No
Wales	7	No	Yes*
	14	Yes*	No
	28	No	Yes
Central	None	None	None
Eastern	40	Yes	No
South West	None	None	None
South East	13	Yes	No
Marches	45	Yes	No
Northern Ireland	44	No	Yes

*The anonymized number ID for the member farms is shown. Yes = at least one dog on the farm tested positive for the test shown. *indicates all samples submitted for the farm tested positive using the given test.*

5.3.7 Farm dog diet

All participants fed their dogs between one to two meals daily, with an almost equal divide between 23 out of 47 participants (51%, 95%CI: 36.7-65.3) feeding one meal and 24 out of 47 (49%, 95%CI: 34.6-63.2) feeding two meals.

Participants were asked to describe the type of diet fed to their dogs in a 'tick-any-that-apply' question format as detailed in Chapter 2 (Appendix III-d). Broad categories of diet included raw meat (flesh) or viscera (offal i.e. liver and/or lungs), further divided into species of origin (sheep, cattle, pig, horse, donkey, goat or poultry) and whether sourced from fallen stock or from a butcher and/or abattoir. Further categories included cooked meat or viscera (from any source), commercial proprietary dog food, catering waste, fish or other (with an open text box for comment for any other category not included).

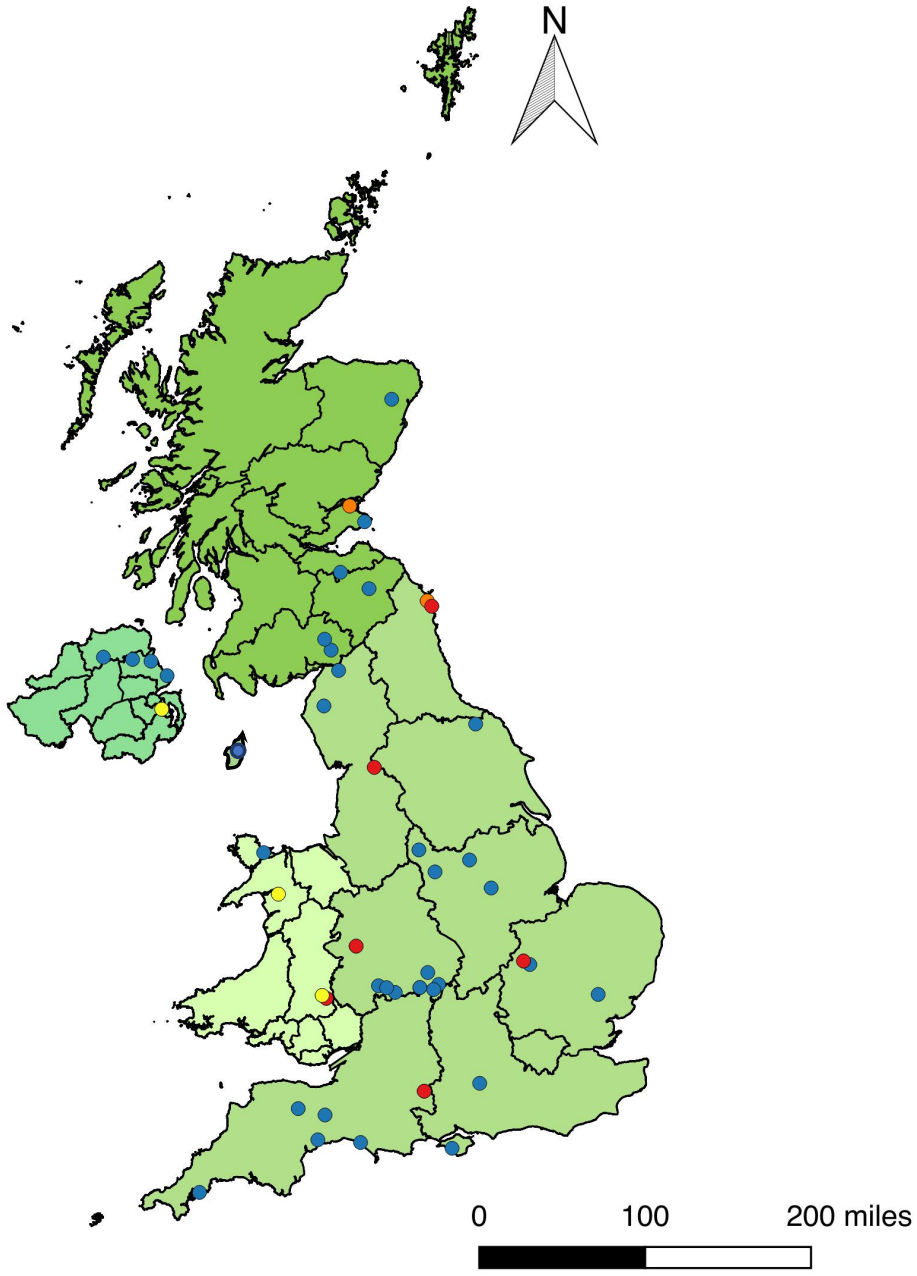

Figure 5-3. Geographical locations and coprodiagnostic test outcome of NSA member farms participating in a UK cross-sectional survey of echinococcosis in farm dogs. Red dots represent farms with at least one farm dog positive on Echinococcus spp. coproELISA only; yellow dots represent farms with at least one farm dog positive on E. granulosus G1 coproPCR; orange dots represent farms with at least one farm dog positive on both tests; blue dots represent farms negative on both tests.

Table 5-6. Summary details of diets fed to farm dogs as part of a cross-sectional survey of 47 NSA member farms. Some farms may be represented in more than one category.

Diet type		Number of farms	%	95% CI
Fallen stock (Meat)	Cattle/Sheep	10	21.2	9.6 - 33.0
	Other mammals	2	4.3	0.0 - 10.0
	Poultry	0	0	0.0
Fallen Stock (Viscera)	Cattle/Sheep	4	8.5	0.0 - 6.3
	Other mammals	1	2.1	0.0 - 6.2
	Poultry	0	0	0
Abattoir/butcher (Meat)	Cattle/Sheep	5	10.6	1.82 - 19.4
	Other mammals	1	2.1	0.0 - 6.2
	Poultry	0	0	0.0
Abattoir/butcher (Viscera)	Cattle/Sheep	5	10.6	1.82 - 19.4
	Other mammals	1	2.1	0.0 - 6.2
	Poultry	0	0	0.0
Cooked (Meat)	Cattle/Sheep	9	19.1	7.9 - 30.4
	Other mammals	3	6.4	0.0 - 13.4
	Poultry	3	6.4	0.0 - 13.4
Cooked (Viscera)	Cattle/Sheep	4	8.5	0.5 - 16.5
	Other mammals	0	0	0.0
	Poultry	0	0	0.0
Other	Commercial	47	100	NA
	Catering waste	23	49	34.6 - 63.2
	Fish	5	10.6	1.8 - 19.4
	Other	4	8.5	0.5 - 16.5

Of farms sampled, 31 out of 47 (66%, 95%CI: 52.4-79.5) fed more than one type of diet to their dogs. All remaining farms, 16 out of 47 (34%, 95%CI: 20.5-47.6), were feeding solely proprietary commercial dog food. All 47 participating farms included proprietary commercial dog food in their dog's diet. Over one third of farms, 17 out of 47 (36.2%, 95%CI: 22.4-50), fed raw animal material sourced from fallen stock to their dogs and 12 out of 47 (25.5%, 95%CI: 13.1-38.0) from abattoirs and/or butchers; 19 out of 47 (40.4%, 95%CI: 26.4-54.4) farms cooked raw food from any source prior

to feeding. Further information on diet by type and species origin is reported in Table 5-6.

A further classification based on putative dietary risk of *Echinococcus* spp. infection was included. Of the farms sampled, 13 out of 47 (27.7%, 95%CI: 14.8-40.4) fed raw viscera from sheep and cattle, classed as very high risk and associated with increased risk of *E. granulosus* infection (Buishi *et al.*, 2006; Carmona *et al.*, 1998; Moro & Schantz, 2009). This increased to 19 out of 47 (40.4%, 95%CI: 26.4-54.4) when including the feeding of raw material from other known viable intermediate hosts of *Echinococcus* spp., classed overall as high risk. No participating farms were feeding solely very high or high-risk material within these two categories. Of the farms sampled, 28 out of 47 (59.6%, 95%CI: 45.5-73.6) were feeding solely foodstuffs considered low risk for *Echinococcus* spp. infection i.e. commercial proprietary dog food, catering scraps, poultry or fish. No participating farms were feeding any material of equine origin, so it was no considered likely that *E. equinus* infection could occur.

With respect to *E. granulosus* routes of transmission, no significant associations were identified between NSA member region and the feeding of very high-risk material (p=0.945) or overall high-risk material (p=0.229). No significant associations were identified between a positive coproELISA result and the feeding of very high risk (p=0.071), high risk (p=0.162) or low risk (p=0.162) diet. Similarly, no significant associations were identified between a positive coproPCR result for *E. granulosus* G1 and the feeding of very high risk (p=0.395), high risk (p=0.194) or low risk (p=0.192) diet. With respect to any positive result on any coprodiagnostic test, no significant associations were identified with the feeding of very high risk (p=0.713), high risk (p=1.000) or low risk (p=1.000) diet.

5.3.8 Storage and disposal of raw animal-based foods

Respondents feeding raw food dietary components to their dogs were asked about the temperature storage methods of the raw foodstuffs. Of 25 respondents indicating feeding raw food, only 11 provided an answer to this question. Of these, 7

(63.6%, 95%CI: 35.2-92.1) stored raw food items in a household freezer, 2 (18.2%, 95%CI: 0-41.0) stored raw food items in a household refrigerator and 2 (18.2%, 95%CI: 0-41.0) stored raw food items at room temperature.

Participants were asked to give information on the methods used for the disposal of fallen stock. The listed options included all types of methods and premises approved under the Animal By-Product Regulations (DEFRA, 2014) for fallen stock collection and disposal. The options of Abattoir and Other (open text box) collection premises were included in the responses, although these options are not permitted routes of disposal. Farmers can arrange ABP collection and disposal by approved premises via the National Fallen Stock Company (NFSCo) or make their own arrangements with government approved ABP premises. Of the farms sampled, 29 out of 47 (61.7%, 95%CI: 47.8-75.6) use the National Fallen Stock Company (NFSCo). Three participants indicated using multiple approved collection premises, one participant indicated using a different disposal method outside of the options given, though declined to name it, and four participants did not provide an answer. Almost half of respondents, 21 out of 43 (48.8%, 95%CI: 33.9-63.8) disposed of fallen stock via knackers yard premises. A total of 8 out of 43 (18.6%, 95%CI: 7.0-30.2) disposed of fallen stock through hunt kennels and of these 5 out of 8 (62.5%, 95%CI: 29.0-96.0) were not doing so via the NFSCo. Further information on fallen stock disposal methods used by participating farms is reported in Table 5-7.

Table 5-7. Summary information of fallen stock disposal methods among NSA member farms participating in a cross-sectional study (43 respondents).

Disposal	Number of farms	Percent	95%CI
Incinerator	5	11.6	2.0-21.2
Rendering plant	7	16.4	5.2-27.3
Knackers	21	48.8	33.9-63.8
Abattoir	0	0	0
Hunt kennel	8	18.6	7.0-30.2
Maggot farm	0	0	0
Zoo	1	2.3	0-6.8
Other	1	2.3	0-6.8

No significant associations were identified between method of fallen stock disposal and a coproELISA positive result (p=0.075) or coproPCR G1 result (p=0.143). Of interest was the finding that one participating farm positive on coproELISA for *Echinococcus* spp. antigen in faeces also supplied fallen stock to a hunt kennel.

5.3.9 Worming of farm dogs

Participants were asked to give information on the type(s) of wormer used in their dogs and the frequency of administration.

The majority of participants, 44 out of 47 (93.6%, 95%CI: 86.6-100), reported regular worming of all dogs on-farm. All respondents were using worming products licensed for use in dogs. A total of 8 different wormer brands were used by respondents, all of them combination anthelmintics with varying treatment coverage for common species of canine intestinal worms. All wormer types were licensed for worming of dogs. Three of the wormers were of the legal category that permits sale only through a veterinary surgeon or with a prescription (POM-V). Of the 8 wormer brands used, 6 contained praziquantel, 1 contained nitroscanate and one contained no cestocidal component. Four products were classified as NFA-VPS i.e. supplied by a vet, pharmacist or specially qualified person (SQP) without prescription and one product as AVM-GSL i.e. available for general sale. No participants were using a product which solely contained praziquantel.

Respondents were asked about where they sought advice on worming products and protocols for their dogs. Over three-quarters of respondents, 36 out of 47 (76.6%, 95%CI: 64.5-88.7), sought information on worming from their veterinary surgeon. Only one respondent indicated that advice was sought from the NSA on worming of their dogs. One respondent indicated that they purchased their wormer from the supermarket and sought no advice on type or frequency of worming. Further details on information source data for worming advice is given in Table 5-8.

Table 5-8. Summary of information sources for worming advice of farm dogs in a cross-sectional survey of 47 NSA member farms.

Information source	Number of farms	Percent	95%CI
Veterinary surgeon	36	76.6	64.4-88.7
Pet shop	6	12.8	3.2-22.3
Online	5	10.6	1.8-19.5
National Sheep Association (NSA)	1	2.1	0 -6.3
Other	2	4.3	0-10
None	1	2.1	0-6.3

Almost all respondents, 46 out of 47 (97.9%, 95%CI: 93.7-100), reported administering wormers to their dogs themselves, with 1 out of 47 (2.1%, 95%CI: 0-6.3) reporting that wormers were administered by a farm worker.

Four respondents did not specify the type or brand of wormer administered to their dogs. The majority of farms, 40 out of 42 (95.2%, 95%CI: 88.8-100), were administering a product containing praziquantel.

There was high variability in the dosage and frequency of wormer administration among respondents, ranging from worming once annually to worming every 6 weeks. There was also considerable variation in worming within and outside of the recommended dose by weight or age of dog, as recommended by some product manufacturers. Taking worming 4 times per year as a baseline minimum (or following product instructions issued to that effect) as advised by ESCCAP for non-risk assessed dogs (ESCCAP, 2017), 24 out of 42 farms (57.1%, 95%CI: 42.2-72.1) used their product according to manufacturer recommendations or the minimum 4 times annually. Of respondents using a wormer containing praziquantel, 23 out of 40 (57.5%, 95%CI: 42.2-72.8) were administering the product at the recommended dose and at a minimum of 4 times per year for tapeworm control in non-risk assessed dogs (ESCCAP, 2017). Only 3 out of 42 farms (7.1%, 95%CI: 0-14.9) administered wormer at the minimum 6-weekly interval recommended by ESCCAP for this *Echinococcus* infection risk group (ESCCAP, 2017).

Importantly, respondents not following manufacturer's instructions or a minimum of 4 times per year when giving a praziquantel-containing wormer were significantly more likely have a dog or dogs test positive for *E. granulosus* G1 on coproPCR ($p<0.05$). All 4 farms testing positive on *E. granulosus* G1 coproPCR were not administering a wormer according to manufacturer's instructions. No significant association was found between the use of a praziquantel containing product and a positive result on any coprodiagnostic test ($p=1$).

On participating farms, the number of days between last worming of dogs on the farm and collection of faecal samples for the study ranged from 0-281 days. As no dogs had received a wormer in the 5 days preceding sample collection, it was assumed the potential for cestode infection at the time of sampling was valid.

5.3.10 Farm dog land access, scavenging of fallen stock and disposal of dog faeces

Participants were asked to give information on sites of farmland access to the farm dogs. More than one response could be entered where applicable. Results suggest that farm dogs have access to multiple sites on-farm, including sites shared with grazing livestock and with public access. Over three-quarters, 36 out of 47 participating farms (76.6%, 95%CI: 64.5-88.7) indicated access of farm dogs to land shared with livestock. Overall, 37 out of 47 farms (78.7%, 95%CI: 67.0-90.4) reported dogs having access to at least one type of land with potential contact with livestock or the public (via open land access). Two participants indicated 'other' land access sites but did not elaborate on what these were. Further details on access to different types of land are detailed in Table 5-9.

Of the 11 farms with at least one dog testing positive on a coproELISA and/or coproPCR test for *Echinococcus* spp., 10 out of 11 farms (90.9%, 95%CI: 73.9-100) had dogs with access to land with shared livestock and/or public access. Overall, land access was not significantly associated with a positive test result ($p=0.414$).

Table 5-9. Summary of land access to farm dogs in a cross-sectional survey of 47 NSA member farms.

Sites of access	Number of farms	Percent	95%CI
Fenced grass yard	16	34.0	20.5-47.6
Fenced concrete yard	13	27.7	14.9-40.4
Fenced field/area with livestock	36	76.6	64.5-88.7
Open land with public access	19	40.4	26.6-54.5
Tethered	2	4.3	0-10.0
Roads	0	0	0
Other	2	4.3	0-10.0

Participating farms were asked to indicate the approximate frequency of witnessing farm dogs scavenge fallen stock. Overall, 21 out of 47 farms (44.7%, 95%CI: 30.5-58.9) reported witnessing their farm dogs scavenging fallen stock on the farm. Frequent scavenging of fallen stock by farm dogs was reported by 4 out of 47 farms (8.5%, 95%CI: 0.5-16.5). Occasional scavenging of fallen stock was reported by 17 out of 47 farms (36.2%, 95%CI: 22.4-49.9), no scavenging of fallen stock was reported by 23 out of 47 farms (48.9%, 95%CI: 34.6-63.2) and one farm, 2% (95%CI 0-6.3), did not know if scavenging of fallen stock took place.

No significant associations were identified between coprodiagnostic test and frequency of witnessing scavenging of fallen stock.

The NSA encourages farmers to place notices advising dog walkers on their farmland to regularly worm their dogs and to pick up and dispose of dog faeces safely. Of the farms sampled, 15 out of 47 (31.9%, 95%CI: 18.8-45.2) indicated that such notices were placed on their land.

Participating farms were asked to give information on collection and disposal of faeces from dogs on farm. Over half (25 out of 47 respondents, 55%, 95%CI: 41.1-69.5) indicated that they regularly collected voided faeces from their farm dogs. Of remaining participants, 19 out of 47 (40.4%, 95%CI: 26.4-54.5) did not collect voided faeces from their farm dogs and 2 out of 47 (4.3%, 95%CI: 0-10) did not know if voided faeces were collected. From those regularly collecting voided faeces, further

information was requested regarding the means of faeces disposal. A summary of disposal means of collected faeces is given in Table 5-10.

Table 5-10. Summary of farm dog faeces disposal means by respondents in a cross-sectional study of 47 NSA member farms.

Faeces disposal site	Number of farms	Percent	%95CI
Rendering plant	0	0.0	na
Muck heap	9	17	7.9 - 30.4
with SRM[a]	0	0.0	na
Burning on site	0	0.0	na
Waste disposal company	1	2.1	0.0 - 6.3
Slurry pit	1	2.1	0.0 - 6.3
Refuse bin	7	14.9	4.7 - 25.1
Buried on farm	3	6.4	0.0 - 13.4
Other	5	10.6	1.8 - 19.5
Don't know	2	4.3	0.0 - 10
Do not collect faeces	19	40.4	26.4 - 54.5
Total	47		

[a]*Specified Risk Material*

Significant associations were identified between dogs on a farm testing positive on *E. granulosus* G1 coproPCR and not collecting faeces at all ($p<0.05$) or not knowing if faeces were collected ($p<0.05$). Very few of the disposal methods used to render faeces potentially infected with *Echinococcus* spp. were safe. Overall, 39 out of 47 farms (83%, 95%CI: 72.2-93.7) disposed of faeces directly on land or by means that could result in the contamination of agricultural or horticultural land if used as compost or fertilizer.

5.4 Discussion

Echinococcus spp. infection at the genus and species level was investigated in farm dogs on 47 randomly selected NSA member farms in the UK. The results of the study indicate *Echinococcus* spp. and specifically *E. granulosus* G1 carriage in farm dogs in many parts of the UK, including novel regions outside the previously identified hot

spot areas. Furthermore, the results also identify a number of husbandry practices that could contribute to transmission of *E. granulosus* in this setting.

5.4.1 Study participation

The study demonstrated that it is possible and practical to undertake a cross-sectional survey of *Echinococcus* in UK farm dogs using a postal questionnaire and sampling approach.

However, the method has some limitations that could introduce biases if the sampling were used to estimate prevalence, such as convenience sampling, variabilities in sample collection and sampling of individual dogs and the small amount of sample material that can be supplied. However, the method required less cost, time and labour than nationwide on-farm sampling and was considered to be appropriate for a study that was looking to provide evidence of presence of echinococcosis in regions of the UK.

5.4.2 CoproELISA and coproPCR results

Overall, 23.9% of participating farms returned faecal samples testing positive for *Echinococcus* on coproELISA and/or coproPCR. CoproELISA assay results identified farm dogs on 17.4% of farms as coproantigen positive for *Echinococcus* at the genus level. Although no comparative studies have been undertaken at the national level, this finding is comparable with coproantigen prevalence rates of 16.2-22% in farm dogs reported in studies in mid-Wales in the last 15 years (Buishi, Walters, et al., 2005; Mastin et al., 2011). Furthermore, this study found a significant association between a positive result on either coprological *Echinococcus* test conducted and farms in the NSA Wales member region. This finding is in agreement with the widely accepted view that Wales remains a focus of echinococcosis in the UK and supports a picture of ongoing active *E. granulosus* transmission in that region. It also provides additional confidence that the results are being interpreted correctly.

Importantly, the study also reports a number of coproantigen and coproDNA positive farm dog samples on farms in other UK regions, reaching far beyond the historical

areas of high prevalence. Coproantigen positive farms are reported here in Eastern Scotland and the Northern, Central and South Eastern regions of England. To the authors knowledge, this study records the first reports of *E. granulosus* G1 by coproPCR in farm dogs in Northern Ireland, Scotland and Northern England. Ireland is believed to be non-endemic for *E. granulosus*, with no autochthonous human cases of cystic echinococcosis reported to date (Deplazes et al., 2017; Torgerson & Budke, 2003). The finding of a farm dog positive for *E. granulosus* G1, a serious zoonotic pathogen, calls for further sampling and information on the dogs on this farm, including history of travel to a known endemic area. Notably, the dogs on the NI farm testing positive had access to land shared with livestock and had been witnessed, on rare occasions, to scavenge on fallen stock. *Echinococcus* spp. studies in definitive and intermediate hosts in Ireland are lacking and the reason why *E. granulosus* is not apparently established, despite free movement of animals between mainland UK and the island of Ireland, remains unknown. It has been hypothesised that the relatively low sheep population in Ireland compared to Wales would reduce the probability of dog-livestock transmission (Torgerson & Budke, 2003).

Although *E. equinus* is endemic in the UK and Ireland (Cardona & Carmena, 2013; Deplazes *et al*., 2017; Lett *et al*., 2018), none was reported in farm dogs in this study. This finding was expected as no participating farms fed any food of equine origin to their dogs.

Due to the very low number of participants, the study could not provide evidence of either freedom from disease in the NSA member regions where no positive results were returned (except at unrealistically high prevalence) or provide estimates of regional prevalence. Future studies would require larger sample numbers for such inferences to be made.

5.4.3 Congruence of coproELISA and coproPCR results

A serial approach to testing of *Echinococcus* in dogs based on primary screening of all samples with coproELISA followed by coproPCR confirmatory testing of positive samples has been suggested as the most practical and cost-effective strategy for

surveillance studies (Christofi *et al.*, 2002; Craig *et al.*, 2015; Giraudoux *et al.*, 2013; Alexander Mathis & Deplazes, 2006). The HyData study found little agreement between the coproELISA and coproPCR positive test results for this sample group (κ=0.1606). Recent studies have found that results do not always correlate when the tests are used in succession (van Kesteren, 2015), due to low egg counts, low worm burdens, pre-patent period and coprophagia (Craig *et al.*, 2015). In this study, it has been possible to undertake both tests. Furthermore, while still a useful assay, in the UK it is important to distinguish between zoonotic and non-zoonotic species when conducting studies. Further factors affecting the interpretation of coproELISA and coproPCR tests across all three canid studies are discussed in Chapter 7.

5.4.4 Farm dog diet

Over a third (36.2%) of farms fed meat and offal from fallen stock, the highest risk material for *E. granulosus* transmission, although a significant association with a positive coproELISA or coproPCR test was not found. A larger study of *Echinococcus* in farm dogs in Wales found a significant positive association between feeding of raw scraps or offal and an *Echinococcus* coproantigen-positive result (Buishi *et al.*, 2005).

Over a quarter of farms sampled (27.7%) purposefully fed raw viscera from sheep and cattle to their farm dogs. This increased to 40.4% of farms feeding raw meat or viscera from any competent host of *E. granulosus*. This finding demonstrates that the practice of feeding high-risk material for *E. granulosus* infection occurs commonly in UK farm dogs. A significant association between a positive result on the faecal tests and this practice was not seen in this study, possibly due to the low sample numbers. Nevertheless, this practice is associated with increased risk of *E. granulosus* transmission (Buishi *et al.*, 2006; Carmona *et al.*, 1998; Moro & Schantz, 2009) and has implications for animal and public health.

Over a third of farms (34%) fed solely commercial proprietary food to their farm dogs, classed in the study as the lowest risk for *E. granulosus* transmission. This food type was not classified as carrying no risk at all, as the questionnaire did not differentiate between cooked or raw commercial proprietary foods. The market for commercially

prepared raw foods has increased over recent years to meet the rising popularity of raw food feeding of pet dogs and cats. Current EU legislation for raw materials used in pet food permits category 3 ABP from EU or permitted non-EU countries for use in raw pet food manufacture (DEFRA & APHA, 2014). Mandatory meat inspection should remove hydatid-affected meat and offal from products suitable for human or pet animal consumption. However, studies on zoonotic cestode pathogens in raw commercial pet food products are lacking. A recent study of zoonotic bacteria and parasite carriage in Dutch commercial raw pet foods found DNA evidence of *Sarcocystis tenella* and *Sarcocystis cruzi*, parasites transmitted via a dog-livestock cycle, in 8/35 (22.8%) of raw commercial products, including category 3 ABP products of beef and sheep origin (van Bree *et al.*, 2018).

Cooking raw meat products (internal temperature of minimum 65˚C for 10 minutes) prior to feeding to dogs has been advised to reduce the risk of *Echinococcus* spp. transmission to dogs (ESCCAP, 2017; The Council of Hunting Associations, 2015; Torgerson, 2014). In this study, 40.4% of farms were cooking raw food products prior to feeding to farm dogs. This is a positive step in reducing infection risk, however information on cooking conditions was not collected, so it cannot be assumed that the practice rendered foodstuffs safe.

With respect to the storage of raw food fed to farm dogs, 63.6% of farms reported keeping raw meat and offal in the freezer. To render viable hydatid cysts non-infective requires a minimum of one week frozen at -17˚C to -20˚C in a typical household freezer (ESCCAP, 2017). Protoscoleces isolated from sheep liver hydatid lesions can remain infective to dogs for up to 3 weeks at temperatures between 0-10˚C (Diker, Tinar, & Senlik, 2008). These findings highlight the importance of advising farmers on optimal storage of raw meat and offal to prevent possible *E. granulosus* infection (although not feeding raw foods would be preferable), together with hygiene and handling advice that would mitigate transmission risk of multiple microbiological pathogens of animal and public health importance (PFMA, 2019).

5.4.5 ABP disposal

Farmers can arrange ABP collection and disposal by approved premises via the National Fallen Stock Company (NFSCo) or make their own arrangements with government approved ABP premises. Over half of participating farms (61.7%) disposed of fallen stock via the NFSCo and 18.6% reported disposing of fallen stock via hunt kennels, although most doing so were not via the NFSCo scheme. In contrast, 48.8% of farms disposed of fallen stock via knackers premises with the majority of farms doing so via the NFSCo. The single farm disposing of fallen stock to zoo premises did so via the NFSCo. Members and collectors of the scheme are required to adhere to biosecurity guidelines alongside those issued by local authorities. The guidelines emphasise moving fallen stock to a designated area that scavenging animals cannot access pending carcase collection (NFSCO, 2015).

Of interest is the finding that one participating farm testing positive on coproELISA for *Echinococcus* spp. antigen also supplied fallen stock to a hunt kennel. A positive coproELISA result in resident farm dog(s) suggests that *Echinococcus* spp. transmission is occurring on this farm. Although this is an indirect inference made without evidence of hydatid disease in the livestock on the farm, if present, it could present a potential route for *Echinococcus* infection to hounds at the hunt receiving fallen stock. This is more notable still, as the farm is in a region outside of known hotspots of *E. granulosus* prevalence in the UK.

5.4.6 Worming of farm dogs

This study found a significant association between suboptimal worming with praziquantel (less than 4 times per year or not according to manufacturer instructions) and a positive result on *E. granulosus* G1 coproPCR ($p<0.05$). This agrees with the findings of Buishi *et al.* (2005) in a study of echinococcosis in Welsh farm dogs, where infrequent worming (<4-month intervals) was a significant risk factor for positive coproantigen results (Buishi *et al.*, 2005). The importance of adequate worming in this setting is also evidenced in a study of Welsh lambs as sentinels for *E. granulosus* infection, which reported the lifting of 6-weekly farm dog worming in the

Wales Hydatid Control Programme in 1989 as responsible for the subsequent rise in prevalence of *Echinococcus* in farm dogs. The authors also proposed that the educational campaign that followed was insufficient to prevent further transmission (Lloyd, Walters and Craig, 1998).

The findings that 93.6% of farms in this study report regularly worming their dogs and of these 95.2% were doing so with a product containing praziquantel are encouraging. However, the finding that only 57.5% of farms using praziquantel were doing so at a minimum 4 times per year, the minimum for non-risk assessed dogs, and only 7.1% at 6-weekly intervals, the recommended amount for this risk group is an important finding of concern.

All farms used worming products licensed in dogs. This is in contrast to the findings of the hunting hound study (Chapter 3), where a number of products, such as ivermectin, were used off-licence as worming treatments.

Given almost all participating farms were worming with praziquantel, it is not surprising that a significant relationship with copro-test positivity was not found. This would suggest that other variables play an important part in the infection dynamics of farm dogs in this setting.

Over three-quarters (76.6%) of farms reported seeking worming advice from their veterinary surgeon and only one indicated seeking advice from the NSA. However, 42.5% of farms not worming appropriately with praziquantel for *Echinococcus* prevention suggests there is scope to better inform veterinary service-providers to farms on the risk-based approach to worming. Despite a growing range of antiparasitic treatments available on the veterinary market, particularly compound endectocidal products aiming at broad parasite cover (National Office of Animal Health, 2017), there is a paucity of research on anthelmintic use in UK companion animal practice. Veterinary electronic data surveillance networks, such as SAVSNET (SAVSNET, 2019) and VetCompass (VetCompass, 2019), offer valuable opportunities to research anthelmintic prescribing practices in the context of *Echinococcus* spp. prevention and use this to target health messages to relevant stakeholders. These

findings also highlight an opportunity for the NSA to increase advice to its members on appropriate worming and other practices to mitigate cestode transmission between dogs and livestock on-farm.

5.4.7 Faeces disposal

In urban areas, local authorities promote responsible disposal of dog faeces by pet owners through legislation, fixed-penalty fines and information campaigns. In England, Wales and Scotland, legislation does not extend to land used for agriculture (Anti-social Behaviour, Crime and Policing Act, 2014). Furthermore, legislation in Scotland specifically removes the requirement for persons to clear up the faeces of working dogs being used for the tending or driving of sheep or cattle (Scottish Government, 2003). A number of campaigns have focused on mitigating the risks associated with dog fouling on farmland. For example, the NSA and National Farmers Union have active anti-fouling campaigns on farmland accessed by the public (NFU, 2019; NSA, 2019a). A study in 2018 by NFU Scotland of 340 farmers and crofters reported 100% of respondents were concerned about dog fouling on their land, with many reporting disease in livestock resulting from ingested faeces and plastic poo bags (NFU Scotland, 2019). In this HyData study, 31.9% of farms reported displaying anti-fouling notices on their land. Importantly, the study also found that 40.4% of farms reported not routinely collecting the faeces of their own farm dogs and 4.3% did not know if faeces were routinely collected. Notably, significant associations were identified between dogs on a farm testing positive on *E. granulosus* G1 coproPCR and not collecting faeces at all ($p<0.05$) or not knowing if faeces were collected on farm ($p<0.05$). Although it may not be practical to always collect the faeces of farm dogs when they are working with livestock, these findings highlight the importance of routine faeces collection when possible. When not possible, it places greater emphasis on regular, optimal worming of farm dogs to mitigate the risk of important helminth and cestode species transmission from dogs to livestock (NSA, 2016, 2019b).

This study found that 83% of participating farms collecting and disposing of farm dog faeces do so via routes that could potentially re-infect the environment should

Echinococcus eggs remain viable within contaminated composted material. Furthermore, participants not collecting faeces or unaware of the collection of faeces on their farm were significantly more likely to have dogs testing positive for *E. granulosus* G1 on coproPCR. However, it cannot be assumed that the presence of coproDNA is indicative of the presence of viable eggs (Alvarez Rojas *et al.*, 2018). An evidence base on the risk of not collecting voided faeces or disposal method of collected faeces on *E. granulosus* transmission to grazing livestock is lacking. Although there is no standardised method for assessing taeniid egg contamination of food samples, studies have reported contamination of fruit and vegetables with *E. multilocularis* and *E. granulosus* (Federer *et al*., 2016; Lass *et al.*, 2015) eggs, presumed to be contamination from voided canid faeces. The significance of disposing dog faeces onto muck heaps or sewage destined as fertilizer for cultivation of fruit and vegetables for human consumption is unknown.

5.4.8 Scavenging

The finding that 44.7% of farms reported witnessing farm dogs scavenging fallen stock on farmland, 8.5% frequently and 36.2% occasionally is important. Opportunistic scavenging on dead livestock carcasses has been suggested as a risk factor for *E. granulosus* carriage in farm dogs, though typically inferred from roaming behaviour, rather than from witnessed scavenging (Palmer & Biffin, 1987; Walters & Clarkson, 1980). When roaming behaviour in farm dogs has been explored as a risk factor for coproantigen positivity, studies estimated roaming dogs to be 2.9 times more likely to be coproantigen positive than chained dogs (Buishi *et al.*, 2005) and 4.9 times the odds of copropositivity if regularly roaming compared to not (Mastin *et al.*, 2011). To the author's knowledge, this is the first study of farm dogs in the UK to gather data on dogs witnessed to scavenge on carcasses of fallen stock. The study did not find an association with scavenging behaviour and a positive test result.

5.4.9 Study limitations

Several limitations relating to the study design need to be acknowledged. The study recruited a much lower than expected number of farms. A two-stage recruitment

process was used, where the NSA initially contacted farms in the first instance, which then contacted the author if they were willing to participate. A better response rate may have been obtained if the author was able to contact farms directly. However, in order to ensure member details remained anonymous unless they were willing to participate, this was the only feasible option for recruitment. As a result, inferential statistical analysis of the resultant dataset was limited. While tests did demonstrate cases in some NSA member regions, the study cannot report freedom from disease in NSA member regions where a positive case was not returned. It is also possible that a number of confounders exist that have not been identified or explored due to this limitation. For example, it is possible that dogs permitted to roam and scavenge fallen stock may be less likely to be adequately wormed (Mastin *et al.*, 2011). It is also possible that significant associations between farm dogs testing positive for *E. granulosus* and a number of important variables were not detected due to the small sample size of the study.

The study used a convenience sampling method. Selecting the study population from the NSA member base could present a potential bias in the representation of sheep farmers owing dogs. The NSA is a membership association established to promote excellence in sheep farming and provides expert advice to its member base, including flock health, farm biosecurity and worming control in dogs (NSA, 2019b). The NSA provides resources advising farmers on disease transmission risks from dog faeces and the scavenging of carcasses on-farm. Furthermore, as participation in the study was voluntary, those taking part may have represented the most pro-active individuals within a membership-base already dedicated to high standards in farming. This may have resulted in an underestimation of potential risk practices to the transmission of *Echinococcus* in UK sheep farms or equally report an overestimation of the understanding of these risks. Compulsory participation studies investigating *Echinococcus* prevalence in farm dogs on a country-wide or regional level, such as those undertaken in Wales (Craig & Larrieu, 2006; Mastin *et al.*, 2011; Palmer & Biffin, 1987) and the Falkland Islands (Lembo *et al.*, 2013) are likely to present a more accurate picture of canine echinococcosis in their study regions.

The NSA does not keep record of which members own working or non-working dogs, so the sample frame could not be recruited solely from the relevant sample base conforming to the inclusion criteria of the study. This may explain in part the disparity between respondents and participants as some respondents did inform us that they did not have farm dogs or no longer kept sheep.

Additional questionnaire information could have contributed to a more holistic analysis involving the other HyData studies. For instance, it would have been useful to gather information on whether abattoir condemnation reports had been received by participating farms relating to cestode parasites with a dog-sheep lifecycle, and whether hunt packs were permitted to cross their land.

5.4.10 Conclusions and recommendations

The current study has identified *E. granulosus* G1 coproDNA in farm dogs in Wales and in previously unreported regions of the UK including Northern Ireland, Eastern Scotland and the North East of England. Confirmatory sequencing of positive samples is needed, alongside further investigation to establish the travel history of dogs testing positive, most importantly in Northern Ireland, where *E. granulosus* is currently thought to be absent. Such investigation should be undertaken in collaboration with the relevant animal health and public health authorities. A significant association was found between *E. granulosus* coproDNA positivity and farm location in Wales, where *E. granulosus* is thought to be re-emergent. Further farms in Wales, England and Scotland, outside of known areas of high prevalence, have dogs testing positive for *Echinococcus* spp. coproantigen. Together, these results suggest that *E. granulosus* is more widespread in farm dogs in the UK than has been reported to date. There is a need for further targeted surveillance studies at the UK-wide level to better understand the significance of the individual cases identified in this study.

Despite considerable advances in accurate *in vivo* diagnosis of echinococcosis in dogs, challenges remain with accuracy and interpretation of coproantigen and coproDNA methods, particularly under conditions where the expected prevalence is

low. Diagnosis and detection using both methods must allow consideration of the strengths and limitations of available tests, together with their labour intensity and cost-effectiveness.

A number of commonly identified risk factors for *E. granulosus* canine infection were reported in this study, including the practice of feeding dogs raw meat and offal, observation of dogs scavenging fallen stock and inadequate worming with praziquantel to control *Echinococcus* egg shedding. The study also highlights a number of practices that could present a potential risk to *Echinococcus* transmission, but where evidence is lacking. Farms are generally not storing raw meat and offal in ways that would destroy infective hydatid cysts. Many farms are not routinely collecting and disposing of farm dog faeces. When faeces are being collected, it is often being disposed of in ways that could potentially re-contaminate grazing pasture with viable eggs, such as muck heaps, slurry pits or on-farm burial, should the environmental conditions favour it. There is a paucity of research on the role and importance of these practices on *E. granulosus* transmission.

The common use of praziquantel wormers in this study group is a positive step towards adequate *E. granulosus* prevention. However, praziquantel was often not administered at a dose and frequency to successfully prevent *E. granulosus* infection and egg shedding. Worming less than 4 times per year was associated with *E. granulosus* coproDNA positivity in this study. Participating farms primarily sought advice on dog worming from their veterinary surgeon. This study highlights the importance of the role of veterinary surgeons and NSA in provision of evidence-based recommendations to prevent *E. granulosus* transmission in farm dogs and discussion of slaughter reports indicating hydatid disease in livestock. The results from this study were reported to participating farms, together with advice that they be discussed with the participant's veterinary surgeon. It is hoped that data generated by the farm dog study will provide valuable information to farm dog owners about preventative health measures and parasite control, and putative public health risks in the event of zoonotic parasite carriage. This information may be used to evaluate and modify existing parasite control measures, contributing to improved animal and

human health. Owners will be encouraged to talk to their vet about positive test findings and to take the study information sheet with them.

This study supports the overarching thesis hypothesis that *E. granulosus* remains a risk to public health in existing UK hotspots, but also provides evidence of a much wider geographical distribution, suggesting both an emergent and re-emergent picture in the UK. These results emphasise the need for up-to-date baseline prevalence data for *E. granulosus* in definitive and intermediate hosts in existing and novel locations, together with a comprehensive assessment of the current risk factors that perpetuate and increase transmission of this zoonotic disease.

Chapter Six

Hydatid disease in cattle in England and Wales

6 Hydatid disease in cattle in England and Wales

6.1 Introduction

Infections with *E. granulosus* occur in a wide range of natural and aberrant intermediate hosts, including many livestock species. Prevalence surveys of hydatidosis in livestock are important to establish transmission levels within and between regions and to investigate the significance of different species in the transmission dynamics of the parasite (Eckert *et al.*, 2001). Through meat hygiene inspection in abattoirs, information can be obtained on geographical distribution (via tracing of animal movements), infection burden relating to age, lesion location and fertility of cysts. However, in some cases, such studies have been limited in, or omitted from disease surveillance efforts, reducing the value of control programmes and limiting their success (Eckert *et al.*, 2002). This chapter describes a multi-site survey of cystic echinococcosis in cattle and sheep in abattoirs in England and Wales. The study uses molecular techniques and histopathology as confirmatory testing to evaluate the meat inspection process and cattle movement records to investigate the origin and movement of infected cattle within and outside known UK hotspots of infection.

6.1.1 Animal health and disease monitoring in abattoirs

6.1.1.1 Meat inspection and CCIR

Ante-mortem and *post-mortem* inspections of animals sent to abattoirs are central to mitigating hazards to public health, animal health and animal welfare. All livestock sent to approved slaughter premises are subject to inspection before meat is sold for human consumption. This is an important aspect of a wider farm-to-fork husbandry process to protect the public from meat-borne zoonotic diseases (AHDB, 2018).

The Food Standards Agency (FSA) is a non-ministerial UK government department responsible for ensuring food safety and hygiene across England, Wales and Northern Ireland, with the remit to ensure meat hygiene in approved abattoirs. Food Standards Scotland (FSS) is the equivalent public food sector body for Scotland. Meat

inspections are carried out by Meat Hygiene Inspectors (MHIs) and Official Veterinarians (OVs) on behalf of the FSA. The responsibilities, tasks and duties of inspectors and veterinary contractors in abattoirs are detailed in the FSA Manual of Official Controls (FSA, 2018b).

The collection of accurate *post-mortem* disease data provides valuable information on the health and welfare of livestock. The data collected should be used as a means to inform and improve farm strategy to reduce losses to the industry (AHDB, 2018; FSA, 2018c; SHAWG, 2019). There is an information cycle that takes place relating to the slaughter of animals. Under the Food Chain Information (FCI) system, the farmer provides information to the abattoir about any animal intended for slaughter and human consumption. This information allows the official veterinarian to assess any potential public health risks relating to the animal and inform any inspection procedures required (FSA, 2018; SHAWG, 2019). After *ante-* and *post-mortem* inspection processes, inspection findings are recorded and reported back to farmers as part of the Collection Communication of Inspection Results (CCIR) process. The flow of information between inspection site and farmer is being developed to inform health and welfare strategy, improve productivity and minimise production losses (FSA, 2018d).

Disease conditions recorded during *ante-* and *post-mortem* inspection often lead to loss in the value of a carcasses (Van Klink, Prestmo and Grist, 2015; AHDB, 2018). In May 2016, the FSA, in partnership with the Agriculture and Horticulture Development Board (AHDB) and the abattoir industry, established a list of *post-mortem* rejection conditions as part of the Better Returns Programme (BRP) (AHDB, 2018). The programme promotes cattle and sheep farmers to improve farming strategy to reduce costs, environmental impact and improve animal performance. The focus is on diseases that are identifiable at slaughter and have existing intervention methods. (AHDB, 2018). Hydatid disease is listed as one of three conditions caused by tapeworms transmissible between dogs and livestock, alongside *Cysticercus ovis* (sheep measles) and *Cysticercus tenuicollis* (sheep bladder worm). The list also includes *Cysticercus bovis* (beef measles), another tapeworm disease with considerable losses to the beef industry (AHDB, 2018). For the purposes

of CCIR reporting, hydatid and other tapeworm lesions are categorized according to the site of organ rejection i.e. head, offal, carcase or total rejection of the animal. As part of CCIR data returned to the farmer, the scheme advises on the risk factors of *E. granulosus* and control measures that should be taken if found on-farm. This includes regular worming of working and visiting dogs with praziquantel, not feeding raw offal to dogs, preventing scavenging of carcases, fencing off public footpaths and encouraging dog owners to pick up their dog's faeces (AHDB, 2018).

6.1.2 *E. granulosus* in UK intermediate livestock hosts

6.1.2.1 Risk factors for infection

Transmission of *E. granulosus* relies primarily on a cycle of infection between domestic dogs as definitive hosts and livestock as intermediate hosts. *E. granulosus* is known to be endemic to the UK, with infection maintained primarily between farm dogs and sheep, through the ingestion of grazing pasture contaminated with the faeces of infected dogs (McManus *et al.*, 1989).

Age has been identified as a significant determinant of CE risk in livestock, with older animals recording higher CE prevalence (Banks *et al.*, 2012) and increased cyst abundance (Ibrahim, 2010). As such, slaughter reports of hydatid disease from abattoirs slaughtering predominantly young animals may underestimate prevalence (Eckert *et al.*, 2002; OIE, 2018). Reports on the significance of gender as a possible determinant of CE vary, although more studies have found female cattle and sheep to be more likely to be infected (reviewed in Otero-Abad and Torgerson, 2013).

6.1.2.2 Disease

In the live animal, depending on the size and location of hydatid cysts, the presence of cysts can affect organ function and lead to poor growth and reduced milk yield, however most infected animals do not show signs of infection (Eckert and Deplazes, 2004; AHDB, 2018). Cysts are typically found in the liver and lungs, though can occur in other organs, such as muscle and bone. Post-mortem, hydatid cysts can reduce the overall value of a carcase through condemnation of affected organs, poor carcase

weight and meat yield and in severe cases, rejection of the whole carcase when hydatid disease has led to emaciation of the animal.

The time period from egg ingestion in intermediate hosts to larval stage infectivity has been reported as up to 2 years with *E. granulosus*, compared with 3 months for *T. hydatigena* and 6-9 months with *T. ovis* (Gemmell, Lawson, & Roberts, 1987).

The rate of development of *E. granulosus* in the intermediate hosts is variable and depends on a number of factors, including the parasite genotype, host species and burden of infection (Thompson, Deplazes, & Lymbery, 2017). The growth rate of hydatid cysts has been reported as 1-5cm per year (Heath, 1973; Thompson, 2017) with viable protoscoleces within brood capsules in sheep identified in between 10 months and 4 years (Gemmell, Lawson, & Roberts, 1986; Heath, 1973). In horses, fertile cysts of *E. equinus* of 2mm in size have been reported as fertile (Edwards, 1981).

Classical hydatid cysts are characterised by a thick, laminated layer surrounding a thin inner layer of cells (germinal layer). In fertile cysts, the germinal layer gives rise to brood capsules containing maturing protoscoleces. Classical cysts are filled with hydatid fluid and when the cyst is fertile, numerous brood capsules and protoscoleces create a gritty substance termed 'hydatid sand' (Agudelo Higuita *et al.*, 2016). Discrete daughter cysts can form within the main cavity to form multivesicular cysts, each containing further brood capsules and protoscoleces. As cysts degenerate, daughter cysts collapse and form infolded and closely packed layers of laminated tissue (Bortoletti *et al.*, 2013). At this stage, cysts are likely no longer viable and can ultimately become caseous and calcified.

The intensity of the host cellular reaction that surrounds the early developing hydatid cyst varies between hosts and can determine whether a viable and infectious cyst develops. In natural hosts, such as horses, the initial reaction resolves, leaving a fibrous host-derived capsule surrounding the viable growing cyst (Ronéus, Christensson, & Nilsson, 1982). If the reaction does not resolve, as has been reported

in cattle infected with *E. granulosus*, it will cause degeneration and eventual death of the parasite (Thompson, 2008).

Global studies have reported high prevalence of infection in cattle (Acosta-Jamett *et al.*, 2014) which are perceived generally as accidental dead-end hosts (Eckert *et al.*, 2002). However, reported studies differ on the fertility of hydatid cysts caused by *E. granulosus* in cattle, with reports ranging from <20%-75% cyst viability (Eckert *et al.*, 2002; Latif *et al.*, 2010).

6.1.2.3 Diagnosis

The presence of a two-host cycle with *E. granulosus* means that epidemiological studies need to consider detection or diagnosis of both adult worms in the definitive host and the larval metacestode stage in intermediate hosts (Craig *et al.*, 2003).

The main method for diagnosis of hydatid disease in intermediate livestock hosts is cyst detection during meat inspection or at post-mortem examination (Craig *et al.*, 2015; Eckert & Deplazes, 2004). During meat hygiene inspection, hydatid cysts are detected in affected organs by observation, palpation and incision of lesions. Affected tissues or organs are removed and condemned or whole carcases condemned, depending on the severity of infection. The reference standard diagnosis for surveys or surveillance is described in the World organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2018). This process involves removal and microscopic examination of cyst contents for the presence of protoscoleces and repeated thin slicing of cyst lesions for gross and histopathological observation of laminar and germinal inner cyst layers characteristic of *Echinococcus* spp. cysts. The laminated layer is a thick, mucin-rich acellular matrix present only in the genus *Echinococcus* and is a diagnostic feature (Eckert *et al.*, 2002; Lin *et al.*, 2013). Fixed sections for histological examination can be stained with periodic acid Schiff (PAS) to visualise the laminated membrane and differentiate it from other liver cysts (Eckert *et al.*, 2002). Sections can also be stained with haematoxylin and eosin (HE) to examine other hydatid cysts characteristics.

Immunohistochemical methods can also improve the accuracy of diagnosis (Craig *et al.*, 2015).

Molecular methods including conventional PCR, Restriction Fragment Length Polymorphism PCR (PCR-RFLP) and quantitative real time PCR have been used to detect and distinguish *Echinococcus* spp. genotypes from cyst material in human and animal intermediate hosts. These have been extensively reviewed in the literature (Carabin *et al.*, 2005; Siles-Lucas *et al.*, 2017; Torgerson & Deplazes, 2009). Several studies have used PCR and DNA typing of species and genotypes in surveys of cysts discovered at slaughter inspection (Bardonnet *et al.*, 2003; Boufana *et al.*, 2014; Casulli *et al.*, 2012; Guo *et al.*, 2019; Siles-Lucas *et al.*, 2017). PCR and direct sequencing of products to determine cox1 and NAD1 gene sequences are considered the best methods for molecular identification of *Echinococcus spp*. and strains (Eckert *et al.*, 2002).

There are no widely applied methods for the *in vivo* detection of infection in intermediate hosts, although ultrasound and serology have been trialled in surveillance studies of small ruminants with reported test sensitivity of 54.4-88.7% and specificity of 75.9-100% (Dore *et al.*, 2014; S. Lahmar *et al.*, 2007; Sage *et al.*, 1998). Serology has been considered a potentially important tool for surveillance of hydatid disease in endemic areas and as part of hydatid control programmes (Craig *et al.*, 2015). In particular, the ability to identify *E. granulosus* in lambs used as sentinels for infection would be especially useful during control efforts (Eckert *et al.*, 2002). Although sheep mount an effective IgG response to *E. granulosus* within weeks (Blundell-Hasell, 1969), resultant serum antibody titres can be variable. Studies evaluating serodiagnostic tests, such as ELISA and immunoblotting have been reviewed in the literature (Craig *et al.*, 2015). Ultrasonography (Dore *et al.*, 2014; S. Lahmar *et al.*, 2007; Sage *et al.*, 1998) and serodiagnosis (Blundell-Hasell, 1969; Ibrahim, 2010), have been used with varied success and limitations in specificity due to co-infection with other taeniid cestodes.

Abattoirs provide a valuable opportunity for surveillance of livestock CE (Craig *et al.*, 2017). Prevalence of hydatidosis in intermediate hosts is strongly age dependent,

and abattoirs slaughtering predominantly young animals may underestimate prevalence rates of infection (Craig *et al.*, 2015; OIE, 2018) as such it is important that surveillance work is stratified according to age (OIE, 2018).

The Cattle Tracing System (CTS) records the identification, birth, movement and death of individual animals in Great Britain. The system allows almost real-time surveillance of animal locations on holdings and the size and composition of the national herd. The equivalent Animal and Public Health Information System (APHIS) is operational in Northern Ireland.

In response to the 2001 Foot and Mouth Disease outbreak, the UK government established a disease surveillance strategy. An outcome of this strategy was the Rapid Analysis and Detection of Animal-related Risk (RADAR). The RADAR system captures cattle identification and movement data from CTS to build a record of movements throughout the animal's life and is used monitor livestock populations to assess the risk of veterinary disease and to control outbreaks (Elliott & Mcdonnell, 2007; Vernon, 2011).

Although the main burden of *E. granulosus* is transmitted via a domestic dog-sheep lifecycle, cattle have been identified in many studies as having the highest prevalence of hydatidosis among the main livestock species (Acosta-Jamett *et al.*, 2014; Deplazes *et al.*, 2017). This finding and the ability to trace movements and identify individual cattle in the UK makes them a good sentinel species for surveillance studies.

6.1.3 Prevalence and distribution of cystic hydatidosis in livestock in the UK

Recent annual Government zoonosis records in Great Britain, based on abattoir surveillance, report 1,315 bovine cases of hydatidosis, based on visible cysts (from 3,676,638 animals slaughtered), 23,596 cases in sheep (from 26,569,918 animals slaughtered) and 13 positive cases in goats (of 7,705 animals slaughtered). In Northern Ireland, two cases of hydatid disease in sheep were reported in an abattoir in 2017 and no cases reported in 2016 (PHE, 2018). Recent CCIR figures published by the FSA for April-June 2019 indicate a prevalence of 0.025% of hydatidosis in all bovine animals (FSA, 2019).

In recent years, surveys in farm dogs suggest a re-emergence of *E. granulosus* in high-prevalence areas of Wales previously targeted by hydatid control programmes (Buishi, Walters, *et al*., 2005; Mastin *et al.*, 2011), raising the concern of an increased infection risk to humans. There is also evidence in livestock and hunting hounds of a wider distribution of *E. granulosus* outside of known high prevalence areas in Wales (Boufana *et al*., 2015; Lett *et al*., 2018; Temple, Jones, & Brouwer, 2013). Recent evidence for a wider UK distribution of hydatidosis in livestock is based on reported disease in cattle at slaughter and the tracing of infected cattle through movement records to many parts of the UK. Analysis of slaughter records of bovine cyst material identified during routine inspection in 19 abattoirs in Wales between 2010-2011, with movement tracing back to birth indicated that hotspots of estimated prevalence remained in mid-Wales. Although not representative for England and Scotland as a whole, further hotspots existed in the midlands of England, Manchester and Perthshire in Scotland (Temple, Jones and Brouwer, 2013). There was a lack of research to support this important evidence with laboratory testing to confirm *E. granulosus* infection. Abattoirs provide an opportunity to collect large amounts of animal health information relevant to the control of zoonotic and notifiable disease. However, the use of such information to inform disease control at the farm level is underutilised (van Klink *et al.*, 2015). There is currently no objective validation of the meat infection process for identification of hydatid lesions in sheep and cattle at slaughter and limited research in this area suggests that meat inspection is highly specific but poorly sensitive in identifying hydatid lesions (Wilson *et al.*, 2019).

6.1.4 Aims

The study had two main aims; firstly, to investigate the hypothesis that hydatidosis occurs beyond the historic areas of high prevalence using slaughtered UK cattle as sentinels. The second aim was to estimate sensitivity and specificity of the meat inspection process against the results of PCR and histopathology of hydatid cysts. It is hoped the results of this work would provide a paradigm on assessing the effectiveness of meat inspection for many other pathological conditions.

6.2 Methodology

6.2.1 Study design

This study was undertaken in collaboration with the FSA and abattoirs in England and Wales. Due to time and resources available, the study did not include the equivalent agencies and abattoirs in Scotland and Northern Ireland, although future studies would aim to do so.

Participant inclusion criteria were abattoir premises in England and Wales slaughtering cattle (with or without slaughtering sheep) and reporting high throughput slaughter figures in the 12 months preceding the study. At the individual animal level, the study included all carcases of cattle and sheep slaughtered for human consumption at FSA-approved abattoirs in England and Wales for which requirements are laid down in Regulation (EC) No 853/2004 (The European Parliament and the Council of the European Union, 2004).

While sheep play a more significant role in the transmission of *E. granulosus* in the UK, cattle were chosen as the predominant species of interest to the study. The accuracy of movement records allows reliable tracing of cattle and build a clearer picture of where transmission is likely to occur (Vernon, 2011).

At participating abattoirs within the sampling period, all lesions identified as hydatid by MHIs on visualization, palpation and/or incision were requested (typically in liver or lung), together with identification data on the affected carcase. In addition, for every hydatid sample, three subsequent samples of *any* identified pathology in liver or lung were collected as negative controls for the identification validation. All samples represented material classified as category 2 ABP for disposal. The samples were returned to the author via Royal Mail First Class post for analysis. Ethical approval (VREC566) for the study was granted by the University of Liverpool Veterinary Research Ethics Committee on 24th August 2017.

The study proposal was also submitted for review by the Institute of Infection and Global Health (IGH) Public Involvement Panel at the University of Liverpool on 18th August 2017. The Panel comprised 20 lay members with varied interests and

backgrounds in areas including animal health and food safety, who offered input to researchers developing project ideas and resources. In particular, the researcher sought feedback on public perception of food safety and animal welfare relating to hydatid disease and the effect upon it should an increased risk of transmission be reported in the UK. Written feedback from the panel was received on 6th September 2017.

Abattoir recruitment commenced on 21st December 2017 and sampling in the first abattoir commenced on 31st January 2018 and the last recruited on 13th April 2018. Sampling completed on 31st April 2019.

6.2.2 Sample size calculations

A database of anonymised weekly slaughter throughput records at all premises meeting inclusion criteria between January 1st, 2015 and 31st December 2015 were provided upon request by the FSA. Additional information included whether located in England or Wales, throughput by species and in cattle, by approximate age i.e. calves <8 months of age and cattle >8 months of age. Only the throughput data for cattle >8 months of age were considered further, as it was thought unlikely that hydatid lesions would have developed to a detectable size by inspection in calves <8 months of age.

Data for cattle >8 months of age were further categorised by annual throughput into high (>10000), medium (>1000 to ≤10000), small (>100 to ≤1000) and very small (≤100). The highest throughput abattoirs accounting for 80% of the total throughput for cattle >8 months of age in England (n=25) and Wales (n=3) were selected for inclusion in the study. Recruitment of high-throughput units would increase the chances of achieving the maximum number of hydatid samples within the timeframe and funds available to the project. In order to calculate apparent prevalence of hydatidosis using the data gathered from the study, CCIR records of reported hydatidosis in cattle and sheep and overall throughput figures for participating abattoirs for the period of study sampling were also requested from the FSA.

Sample size was calculated using estimates for true prevalence (TP), sensitivity (Se) and specificity (Sp). The values selected (TP= 0.02, Se= 0.85, Sp= 0.95) and the resultant sample size of 400 hydatid samples kept the standard deviation of the estimated Se and Sp to a minimum and were realistic within the timeframe and funds of the project.

6.2.3 Collaboration with FSA and abattoir recruitment

Site anonymity was maintained until a selected abattoir agreed to participate in the study. Once the author returned a list of selected anonymised sites to the FSA, the FSA regional Inspection Team Leader (ITL) overseeing operations for each selected abattoir contacted the site to provide project details and invite participation. If this was declined, no further action was taken. Upon agreement to participate, the ITL contacted the author with the location of the abattoir and details of a contact person, usually the Official Veterinarian (OV) or the Operations Manager on site. The author would then discuss the project with the contact person via telephone or email and arrange delivery of the sampling kits and participant information (Appendix IV-a). Whenever possible, the author arranged a meeting with the ITL and abattoir personnel on-site to deliver sampling kits and discuss study logistics in person. When this was not possible, sufficient sampling kits and instructions were sent by courier to the site and receipt confirmed by telephone or email.

Under the terms of a Data Sharing Agreement established with the FSA, project outcome data shared with the FSA would be presented as anonymised summary statistics and would not represent raw data on a named abattoir basis. Hydatid data would represent a proportional figure based on test results and apparent prevalence calculated using throughput data matching the timescale of the study. No information was collected on individual inspectors submitting samples.

6.2.4 Sampling kit design

A sampling kit was designed to allow collection of four samples (one hydatid lesion and three subsequent ‘control’ lesions) and recording of identification on species, lesion location and animal identification (cattle passport number or sheep ear tag

number) for the hydatid sample collected. The design of the kit had three key requirements; firstly, to allow safe and rapid collection of tissue and data recording by MHIs with minimum disruption to the inspection process; secondly, to maintain sufficient sample volumes of one hydatid sample plus three 'control' samples in a viable condition for analysis using packaging material compliant with UN3373 and P650 packaging for Category B biological material (Department for Transport, 2012); thirdly, to source sampling materials within the available budget of the project.

The sampling protocol and kit design were appraised and approved by both FSA Health and Safety and FSA Operational departments. With assistance from the FSA, a prototype kit was tested for one week on the inspection line of an abattoir not participating in the study. Further improvements to the kit were made based on feedback from MHIs, including box design, sample pot labelling and data recording points, to facilitate ease of sampling during the busy inspection process.

Each sampling kit (Figure 6-1) contained four 60ml polystyrene screw top sample containers able to withstand pressure of 95 kPa [0.95 bar]. Data relating to the organ, species and identification of the animal were entered into a space provided on the inside lid of the sample box (Figure 6-2). The box (194x125x68mm), including secondary packaging compliant with UN3373 and P650 packaging for Category B biological material (Department for Transport, 2012) (Alpha Laboratories, UK). The kit also contained a permanent marker and an insulated foil bag to help maintain samples as fresh as possible during transit. Modifications to accommodate the sampling pots and additional items within the box were made by the author.

It was anticipated that hydatid lesion collection and tissue handling to cut samples to a suitable size could require cutting of hydatid cysts and other pathological tissue. Each abattoir was provided with several pairs of safety glasses tested and approved under EN166 (EU) government standards for use at the discretion of the participating MHIs.

Illustrated step-by-step sampling instructions were also provided as a poster for display in MHI offices or communal areas at participating abattoirs (Appendix IV-b).

Collected samples were packaged according to these instructions and the self-addressed box, with pre-paid postage, was returned by Royal Mail First Class post to the researcher. Upon arrival, the sample was either immediately processed, stored at 4˚C if due to be processed no later than the following day, or frozen at -20˚C for processing at a later date.

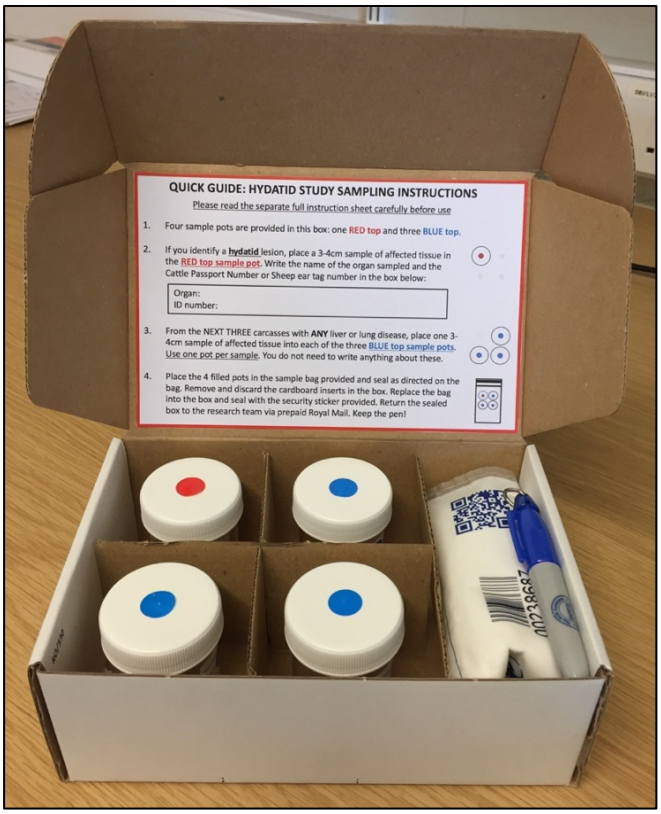

Figure 6-1. HyData sampling kit for collection of hydatid lesions and test control lesions from cattle and sheep during post-mortem meat hygiene inspection.

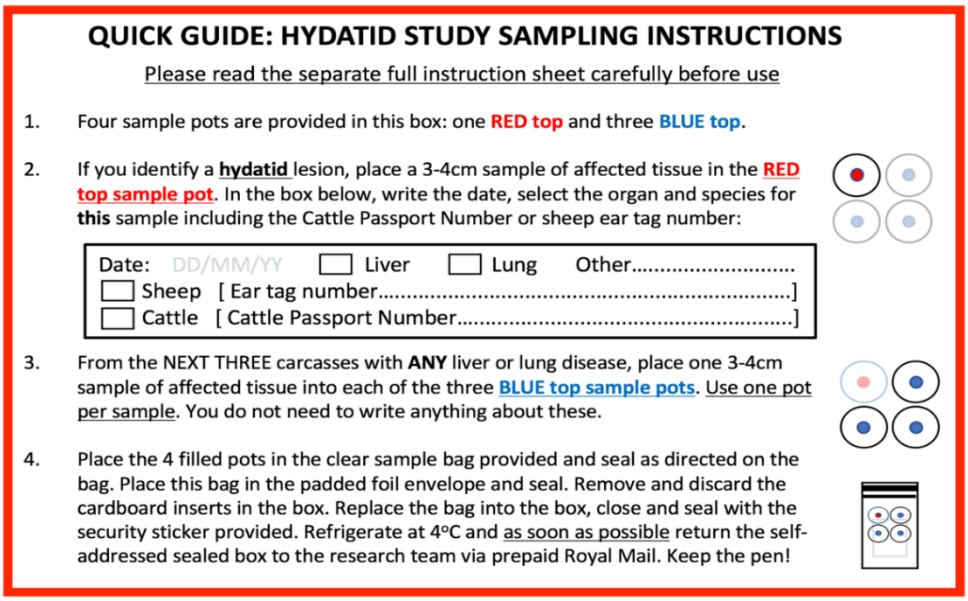

Figure 6-2. HyData sampling kit for collection of hydatid lesions and test control lesions from cattle and sheep during post-mortem meat hygiene inspection.

6.2.5 Sample processing, DNA extraction, PCR amplification and amplicon sequencing

6.2.5.1 Sample processing

On sample arrival, any leakage of sample material in the sample box was scored and recorded as follows: 0= no leakage; 1=leakage on sample pot; 2= leakage into secondary packaging bag; 3= leakage outside of secondary packaging bag; 4= leakage outside of tertiary packing box.

Each sample was handled and dissected separately with sterile single-use or autoclaved instruments to reduce potential cross-contamination. Hydatid lesions were photographed and their gross dimensions and appearance described. If a cyst was presented intact, any cyst fluid was collected with an 18G, 1.5” sterile needle and 5ml syringe. Individual autoclaved Metzenbaum dissecting scissors, DeBakey forceps and disposable sterile single-use scalpels were used for tissue collection from each sample. A ~25mg sample of tissue (approximately a 2mm-sided cube, the recommended amount of stating material for DNA extraction) was collected in duplicate from representative parts of the hydatid cyst or ‘control’ lesions. With

hydatid samples, this included the lining of the inner cyst wall and a sample of cyst fluid if present.

Positive control material for PCR from *post-mortem* samples stored at -20˚C of a hydatid cyst (*E. granulosus*) in bovine liver; *C. bovis* (*T. saginata*) in bovine heart muscle; *C. tenuicollis* (*T. hydatigena*) in ovine liver and *C. ovis* (*T. ovis*) in ovine heart muscle were provided by The University of Liverpool Institute of Veterinary Science. The identity of samples had been confirmed on *post-mortem* examination by a lecturer in Veterinary Public Health at the Institute.

6.2.5.2 DNA extraction and PCR

Genomic DNA was extracted from all duplicate study samples and positive control material using QIAGEN® DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Eluted total DNA was stored at -20˚C until further analysis.

DNA samples were first analysed for genus-specific *Echinococcus* spp. and other cestode spp. using the multiplex protocol developed by Trachsel, Deplazes and Mathis (2007), which amplifies a 267bp fragment of small subunit ribosomal DNA (*rrn*S) from *Taenia*, *Mesocestoides*, *Dipylidium* and *Diphyllobothrium* spp; a 117bp *rrn*S fragment from *E. granulosus* and a 395bp NADH dehydrogenase subunit 1 (*nad*1) fragment from *E. multilocularis* mitochondrial gene (Trachsel et al., 2007b). The protocol was modified to include only primer pair selections amplifying the 117bp *E. granulosus* DNA fragment (*Cest 3* and *Cest 5*) and the 267bp Taenid DNA fragment (*Cest 4* and *Cest 5*). The primer pair (*Cest 1* and *Cest 2*) designated to amplify the 395bp DNA fragment from *E. multilocularis* was omitted. PCR reactions were conducted in a 40µl reaction volume comprising 10µl of template DNA prepared with 5x FirePol® Ready To Use Master Mix (Solis Biodyne) containing 7.5mM $MgCl_2$, 100pmol of forward primers *Cest* (5'-YGAYTCTTTTTA GGGGAAGGTGTG-3') and *Cest 4* (5'- GTTTTTGTGTGTTACATTAATAAGGGTG-3') and 200pmol of the shared reverse primer *Cest 5* (5'-GCGGTGTGTACMTGAGCTAAAC-3') (suspended in 1µl of PCR grade water), supplemented with PCR grade water to a total 40µl. Thermal cycling

conditions were as follows: an initial denaturation step of 5 minutes at 95˚C, followed by 30 cycles of 30 seconds at 95˚C, 1 minute at 56˚C and 40 seconds at 72˚C and a final elongation step of 10 minutes at 72˚C (Trachsel et al., 2007b). For preparation of DNA amplicons suitable for sequencing of *Taenia* spp. isolates only, the assay was repeated using the reverse sequencing primer *Cest* 5*seq* instead of *Cest* 5, as per the published protocol (Trachsel et al., 2007b).

For confirmation and to amplify a sequence with sufficient polymorphic sites to differentiate *Echinococcus* species via sequencing, samples were further analysed using the protocol by Bowles, Blair and McManus (1992) to amplify a ~450bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (*cox*1) (Bowles *et al.*, 1992). PCR was performed in a 40µl reaction volume comprising 10µl of template DNA prepared with 5x FirePol® Ready To Use Master Mix (Solis Biodyne) containing 7.5mM $MgCl_2$, 100pmol of forward primer *JB3* (5'-TTTTTTGGGCATCCTGAGGTTTAT-3') and reverse primer *JB4.5* (5'-TAAAGAAAGAACATAATGAAAATG-3'), supplemented with PCR grade water to a total 40µl. Thermal cycling conditions were as follows: an initial denaturation step of 5 minutes at 95˚C, followed by 30 cycles of 40 seconds at 95˚C, 1 minute at 54˚C, 1 minute at 72˚C and a final elongation step of 10 minutes at 72˚C.

All PCR reactions were performed in an Applied Biosystems 2720 Thermocycler (Applied Biosystems, UK). PCR grade water (Sigma Aldrich, UK) at equal volume to template DNA was included in every reaction as a non-template control. All PCR primers were synthesized by Eurofins MWG Operon, Germany. PCR products were resolved at 110V on a 1.5% or 3% (w/v) Agarose gel (Biogene Ltd., UK) in 1x Tris-Acetate-EDTA buffer (Sigma-Aldrich, UK) stained with PeqGREEN dye (VRW Peqlab, USA). Fragment band size was estimated by comparison with a 100bp DNA ladder (Solis Biodyne, UK) or a Low Molecular Weight DNA Ladder (New England Biolabs inc., USA) depending on anticipated amplicon size(s). Gels were visualized on a UVITEC Gel Documentation System (UVITEC, UK) using UVIProMV v11.02 software (UVITEC, UK). PCR products were commercially purified and sequenced in both directions (Source Bioscience, Nottingham, UK).

6.2.6 Histopathology

A 2mm slice of tissue representative of the hydatid lesion was trimmed into processing cassettes and placed in 10% neutral buffered formalin to fix for a minimum 48 hours. One of the three accompanying 'control' samples was selected using a random number generator in Microsoft Excel 2010 (Microsoft Corporation, USA) to undergo the same process. Tissue cassettes were placed in a vacuum infiltration processor overnight before being embedded in paraffin (Ultra-premium embedding medium, Solmedia); 4µm paraffin sections were cut on a Leica RM2125 RT microtome, floated on a water bath and placed on colour slides (MSS54511YW, Solmedia). For haematoxylin and eosin (H&E) staining, slides were dewaxed in xylene and rehydrated through descending grades of ethanol (100%, 96%, 85%, 70%) to distilled water before being stained in haematoxylin (5 mins) and eosin (2 mins). For Period Acid Schiff staining, slides were similarly dewaxed then placed in 0.5% periodic acid (HC6455-50, TCS Biosciences Ltd) (5 mins) then schiff (HST206-D, Solmedia) (30 mins), before washing in running tap water and staining in Mayers haemalum (HST011, Solmedia) (2 mins). After a 5 min rinse in tap water, all slides were then dehydrated through 96% and 100% ethanol to xylene and cover slipped using DPX mounting medium (Thermo Scientific, Lamb/DPX). Tissue processing for histopathology was performed at the University of Liverpool Veterinary Laboratory Services. Identity of hydatid lesions was reviewed by a board-certified veterinary pathologist at the University of Liverpool.

6.2.7 Cattle Tracing System (CTS) records

In order to explore the hypothesis that hydatidosis was occuring in cattle outside of known areas in the UK, all cattle ear tag numbers associated with the hydatid samples collected were collated and submitted via request to APHA for a RADAR report to include birth, death and movement records (to county level), cattle breed and sex data. The report was requested on 17th April 2019 and received on 1st May 2019.

6.2.8 Analysis

Descriptive data are presented as proportion, percentage and 95% Confidence Interval (95%CI) of the percentage. For analysis of categorical variables a χ^2test and odds ratio statistic were used to investigate associations between a positive *E. granulosus* test result and variables including location of travel (county), cattle breed, purpose and sex. Movement records traced individual cattle movements to county level from birth to slaughter. For test of association with a positive *E. granulosus* test result cattle movement criteria were classified as either having been present in Wales or not present in Wales during any movements recorded in their lifetime. A two-sided Fisher's exact test was used where expected values within variables categories was less than 5. Significance was set at $p<0.05$.

For assessment of agreement between meat hygiene inspection, coproPCR and histopathology dichotomous test results, a McNemar's test was initially used to establish that tests classified approximately the same portion of dichotomous results as positive, with significance set at $p<0.05$. A Cohen's Kappa statistic (κ) was then used to measure the level of agreement between test results at the individual animal level.

For the additional aim of the study, to evaluate the *Se* and *Sp* of meat hygiene inspection for hydatid cysts, a 2-stage approach using a genus-specific *Echinococcus* spp. cox1 PCR as a designated gold standard reference test was used (Dohoo, 2010; Reichenheim & Ponce de Leon, 2002). The equation is shown with relevant values entered in section 6.3.4.1. All statistical analyses were performed using Stata 14 (StataCorp, 2015).

6.3 Results

6.3.1 Study participation

A total of 46 abattoirs, classed by the study as large throughput i.e. with >10000 cattle >8 months old slaughtered annually, were identified in the anonymized list of sites provided by the FSA. The 25 abattoirs with the highest throughput in this category (ranging between 28883 – 190715 cattle >8 months old/year) were selected for recruitment to the study. Following initial contact via FSA ITLs, 15/25 abattoirs agreed to participate in the study, giving a 60% (95%CI: 40.8-79.2) participation rate. Of these, 14 out of 15 were in England (93.3%, 95%CI: 80.7-100.0) and 1 was in Wales (6.7%, 95%CI: 0.0-19.3); 9 out of 15 sites (60%, 95%CI: 35.2-84.8) slaughtered cattle and 6 (40%, 95%CI: 15.2-64.8) slaughtered both cattle and sheep. According to DEFRA data on cattle and sheep slaughter between 2014-18, the average number slaughtered was 1,786,159 cattle and 12,954,899 sheep (DEFRA, 2019). During the period of sampling, the total throughput in the participating premises (n=15) was 1,048,633 cattle (S. Crookes, FSA, pers. comms.) representing 58.71% of the average throughput of all cattle slaughtered in England and Wales during the last 5 years and 902,660 sheep or 6.97% of the average throughput of sheep slaughtered in England and Wales.

A total of 87 samples, identified as hydatid on meat hygiene inspection were returned to the study, along with 261 control samples of other pathologies, from 11 abattoirs in England and Wales. The remaining 4 participating abattoirs did not submit samples to the study. Hydatid samples received from cattle represented 77 out of 87 samples (88.51%, CI: 79.72 - 93.78%), of which 62 out of 77 (80.52%, 95%CI: 70.04 – 87.96 %) were located in the liver, 13 were identified in the lung (16.88%, 95%CI: 10.01 – 27.06%) and 2 (2.6%, 95%CI: 0.64 – 9.92%) in muscle; 10 samples were received of ovine origin (11.49%, 95%CI: 6.26 – 20.16%) and all were detected in offal. A summary of cyst location by species and organ/tissue is shown in Table 6-1.

Table 6-1. Sites of hydatid lesions in sheep and cattle (including number of associated control samples) reported on meat hygiene inspection at participating abattoirs submitted to the HyData project.

	Cattle			Sheep			Overall		
Lesion	Hydatid +ve		-ve	Hydatid +ve		-ve	Hydatid +ve		-ve
	n	% (95%CI)	n	n	% (95%CI)		n	% (95%CI)	n
Liver	**62**	80.52 (70.04 – 87.96)	186	**9**	90.00% (50.30 – 98.77)	27	**71**	81.61 (76.41 – 85.87)	213
Lung	**13**	16.88 (10.01 – 27.06)	39	**1**	10% (12.56 – 49.70)	3	**14**	16.09 (9.72 – 25.46)	42
Muscle	**2**	2.6 (0.64 – 9.92)	6	**0**	0	0	**2**	2.30 (0.57 – 8.83)	6
Total	**77**	88.51 (79.72 – 93.78)	231	**10**	11.49 (6.26 – 20.16)	30	**87**		261

When compared with the CCIR reports of recorded hydatidosis submitted by participating abattoirs matching the time period of the study, the number of samples returned represented 87 out of the 715 reported across all participating abattoirs (12.17%, 95%CI: 9.77 14.56%). For individual participating abattoirs, hydatid samples returned as a percentage of hydatid cases reported by CCIR ranged from 0-128%, where the highest value represented an abattoir submitting more samples than were recorded by CCIR. Four participating abattoirs did not submit any samples to the study; one of these had not reported any cases of hydatidosis in sheep or cattle; two had reported cases, but not submitted samples and CCIR records were not available for the fourth. A summary of hydatid sample returns and CCIR records by species and lesion site for each participating abattoir is shown in Table 6-2.

Table 6-2. Summary of CCIR hydatid disease reporting data and samples received from participating abattoirs during the HyData sampling study

Abattoir Code	Species	Start sampling	End sampling	CCIR Data hydatid lesions							HyData hydatid lesions								Returns	
				Sheep			Cattle			All	Sheep			Cattle				All		
				Offal	Carcase	Total	Offal	Carcase	Total	Total	Liver	Lung	Total	Liver	Lung	Muscle	Total	Total	%	95%CI
0	Cattle	31.1.18	31.1.19	-	-	-	58	4	62	62	-	-	-	6	1	1	8	8	12.90	(4.56 - 21.25)
1	Cattle	28.2.18	28.2.19	-	-	-	8	0	8	8	-	-	-	2	0	1	3	3	37.50	(3.95 - 71.05)
2	Cattle, Sheep	22.2.18	28.2.19	44	60	104	8	1	9	113	3	1	4	2	0	0	2	6	5.31	(1.18 - 9.44)
3	Cattle, Sheep	27.2.18	28.2.19	97	9	106	45	10	55	161	0	0	0	0	0	0	0	0	0.00	
4	Cattle	27.2.18	28.2.19	-	-	-	0	0	0	0	-	-	-	0	0	0	0	0	0.00	
5	Cattle	1.3.18	28.2.19	-	-	-	51	0	51	51	-	-	-	5	1	0	6	6	11.76	(2.92 - 20.61)
6	Cattle	9.3.18	31.3.19	-	-	-	6	0	6	6	-	-	-	0	0	0	0	0	0.00	
7	Cattle, Sheep	14.3.18	31.3.19	ND	ND	ND	42	0	42	42	6	0	6	0	0	0	0	6	14.29	(3.70 - 24.87)
8	Cattle, Sheep	14.3.18	31.3.19	2	2	4	4	9	13	17	0	0	0	1	0	0	1	1	5.88	(0.00 - 17.07)
9	Cattle, Sheep	14.3.18	31.3.19	ND	ND	ND	70	5	75	75	0	0	0	17	0	0	17	17	22.67	(13.19 - 32.14)
10	Cattle, Sheep	22.3.18	31.3.19	0	0	0	7	0	7	7	0	0	0	5	4	0	9	9	128.57	
11	Cattle	22.3.18	31.3.19	-	-	-	2	0	2	2	-	-	-		0	0	0	0	0.00	
12	Cattle	23.3.18	31.3.19	-	-	-	43	0	43	43	-	-	-	14	3	0	17	17	39.53	(24.92 - 54.15)
13	Cattle	23.3.18	31.3.19	-	-	-	87	0	87	87	-	-	-	6	2	0	8	8	9.20	(3.12 - 15.27)
14	Cattle	13.4.18	31.4.19	-	-	-	33	8	41	41	-	-	-	4	2	0	6	6	14.63	(3.82 - 25.45)
			Total	**143**	**71**	**214**	**464**	**37**	**501**	**715**	**9**	**1**	**10**	**62**	**12**	**2**	**77**	**87**	**12.17**	**(9.77 – 14.51)**

The period of time from sampling to kit delivery ranged from 1-17 days, with a median of 3 days (inter-quartile range= 3); 55 out of 87 (63.20%, 95%CI: 53.1– 73.4%), were delivered within 3 days of sampling, increasing to 80/87 (92.0%, 95%CI: 86.2-97.70%) delivered within 1 week of sampling. The storage method used for samples prior to postage was not recorded.

On arrival, sample kits were examined for any leakage of contents; 16 out of 87 kits (18.39%, 95%CI: 10.25 – 26.56%) showed no leakage of contents; 63 out of 87 kits (72.41%, 95%CI: 63.02 – 81.81%) showed blood leakage/contamination on the outside of the sample pots; 4 kits (4.60%, 95%CI: 0.10 - 9.0%) showed blood leakage/contamination into the secondary packaging bag containing absorbent material for this purpose; 2 kits (2.3%, 95%CI: 0.0 - 5.45%) had leakage extending to the inside of the tertiary carboard box packaging.

6.3.2 PCR

6.3.2.1 Multiplex genus-specific cestode PCR and DNA sequencing

A total of 23/323 samples (7.12%, 95%CI: 4.32 – 9.93%) tested positive for *Echinococcus* spp. by amplification of a 117bp fragment of the small subunit of ribosomal RNA (*rrnS*); 4 out of the 23 samples (17.39% 95%CI: 1.9 – 32.88%) were submitted as “controls”. A further 7 out of 324 samples (2.16%, 95%CI: 0.58 – 3.74%) tested positive for *Taenia* spp. by amplification of a 267bp fragment of *rrnS*. A representative agarose gel image demostrating examples of both PCR products is shown in Appendix IV-ci.

No sheep samples tested positive for *Echinococcus* spp. DNA. No cattle samples tested positive for *Taenia* spp. DNA. Within hydatid samples submitted from cattle, 23 out of 285 (8.07%, 95%CI: 4.91 – 11.23%) tested positive for *Echinococcus* spp. DNA. Within hydatid samples submitted for sheep, 7 out 38 (18.42%, 95%CI: 6.1 – 30.75) tested positive for *Taenia spp*. DNA. Results are presented in Table 6-3.

Forward and reverse sequencing of PCR products corresponding to *Taenia* spp. showed 100% sequence identity with *T. hydatigena* mitochondrion complete genome (NCBI Accession No. GQ228819.1) (Jia *et al.*, 2010) in 6 of the 7 isolates. One

isolate did not produce a sequence of sufficient quality for a NCBI database query. All isolates confirmed as *T. hydatigena* by this BLAST search on the NCBI database were from the same abattoir (Abattoir number 7). Due to the small size of the PCR amplicon and limited funds, sequencing for *Echinococcus* spp. DNA products was only undertaken on products from the second PCR in this study.

Cohen's Kappa statistic to assess the agreement of MI and the multiplex genus-specific PCR beyond chance showed fair agreement between the tests (κ = 0.2609, p<0.0001).

Table 6-3. Multiplex genus specific PCR results against meat Inspection. Included are percentage values and Cohen's kappa for level of agreement between tests. Cattle specific results are also included

		Multiplex Genus specific cestode *rrnS* PCR						
		Cattle			All animals			Cohen's κ
		+ve	-ve	Total	+ve	-ve	Total	
Meat Inspection	+ve	**19** 82.61* 25.33	**56** 21.39 74.67	**75** 26.32 100.00	**19** 82.61 22.35	**66** 22.00 77.65	**85** 26.32 100.00	*(p<0.0001)*† **κ = 0.2609** (0.0408) P<0.0001 **Fair**
	-ve	**4** 17.39 1.90	**206** 78.63 98.10	**210** 73.68 100.00	**4** 17.39 1.68	**234** 78.00 98.32	**238** 73.68 100.00	
Total		**23** 100.00 8.07	**262** 100.00 91.93	**285**	**23** 100.00 7.12	**300** 100.00 92.88	**323**	

**Percentage values are included in a column/row format*

†Listed in order: McNemar's p=value (italics), κ value (standard error), κ p=value, level of agreement.

6.3.2.2 Mitochondrial cox1 gene PCR and DNA sequencing

A total of 23/324 samples (7.1%, 95%CI: 4.3-9.1) submitted as hydatid on meat hygiene inspection tested positive for *Echinococcus* spp. by amplification of a ~450bp fragment of the mitochondrial *cox*1 gene. A representative agarose gel image is shown in Appendix IV-cii. Within hydatid samples submitted from cattle, 23 out of 285 (8.07%, 95%CI: 4.91 – 11.23%) tested positive for *Echinococcus* spp. DNA. No samples from sheep tested positive. Results are summarised in table Table 6-4.

Cohen's Kappa statistic to assess the agreement of MI and the genus-specific PCR beyond chance showed fair agreement between the tests (κ = 0.3984, p<0.0001).

The sequence quality of the 23 products was variable. Forward and reverse sequencing of PCR products corresponding to *Echinococcus* spp. showed a sequence identity with *E. granulosus* mitochondrion complete reference genome (NCBI Accession No. NC_044548) (Kinkar *et al.*, 2019) of 99.0-100% in 13 of the products and 86.8-98.9% in 9 products. In all these cases, a BLAST search returned *E. granulosus* mitochondrial cox1 complete or partial gene sequences as the top 20 matches. The quality of the remaining 3 product was too poor to confirm them as *E. granulosus* on a BLAST search and the results were overall non-specific for other sequences.

Table 6-4. Genus specific Echinococcus spp. PCR results against meat Inspection. Included are percentage values and Cohen's kappa for level of agreement between tests. Cattle specific results are also included

		Genus specific *Echinococcus* spp *cox 1* PCR						
		Cattle			All animals			Cohen's κ
		+ve	-ve	Total	+ve	-ve	Total	
Meat Inspection	+ve	**23** 100.00 30.67	**52** 19.85 69.33	**75** 26.32 100.00	**23** 27.6 100.00	**62** 72.94 20.60	**85** 100.00 26.23	(p<0.0001) **κ=0.3984** (0.0432) P<0.0001 **Fair**
	-ve	**0** 0.00 0.00	**210** 80.15 100.00	**210** 73.68 100.00	**0** 0.00 0.00	**239** 100.00 79.40	**239** 100.00 73.77	
Total		**23** 100.00 8.07	**262** 100.00 91.93	**285**	**23** 7.10 100.00	**301** 92.9 100.00	**324**	

**Percentage values are included in a column/row format*

†Listed in order: McNemar's p=value (italics), κ value (standard error), κ p=value, level of agreement.

6.3.3 Cyst morphology and histopathology

A total 87 samples submitted as hydatid on MI were photographed, and their morphology recorded on arrival. All lesions submitted were less than 6cm diameter and were either intact or had been incised as part of the meat hygiene inspection process. Variability was observed in the structure of the submitted hydatid lesions; 87 cysts were examined using published morphological and structural descriptors of

hydatid cysts (Bortoletti *et al.*, 2013) as a guide; 14/77 were hyperlaminated gelatinous cysts (16.1%, 95%CI: 8.4-23.8) (example Figure 6-3) and 57/87 were unilocular cysts (65.5%, 95%CI: 55.5-75.5%) (example Figure 6-4).

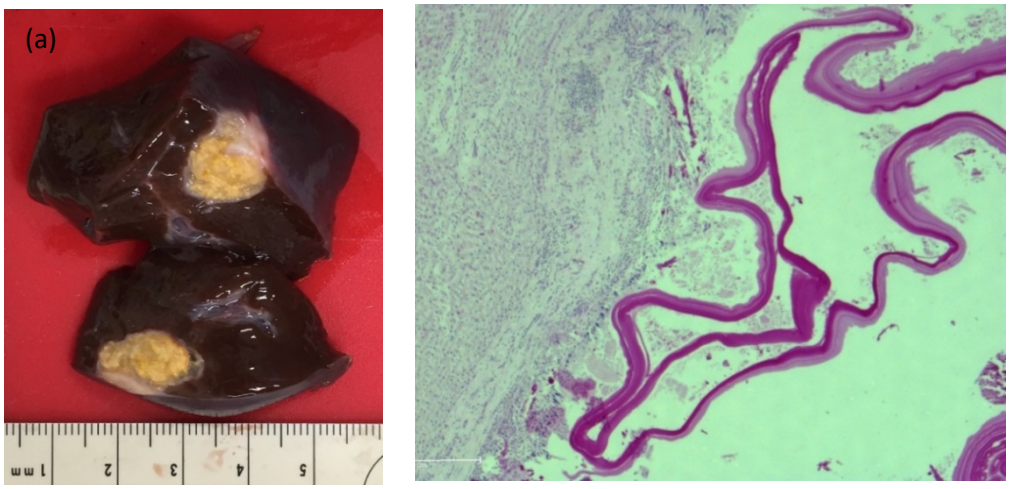

Figure 6-3. Example of an incised hyperlaminated gelatinous hydatid cyst in bovine liver, identified at abattoir meat hygiene inspection and submitted to the HyData study. The cyst lumen in filled with compacted sheets of laminar tissue, with no fluid in the cell interior. b) The laminar membrane is visible on histopathology, deeply stained with PAS stain (x4 magnification). E. granulosus was confirmed by PCR and DNA sequencing. The gross image is shown against a cm scale.

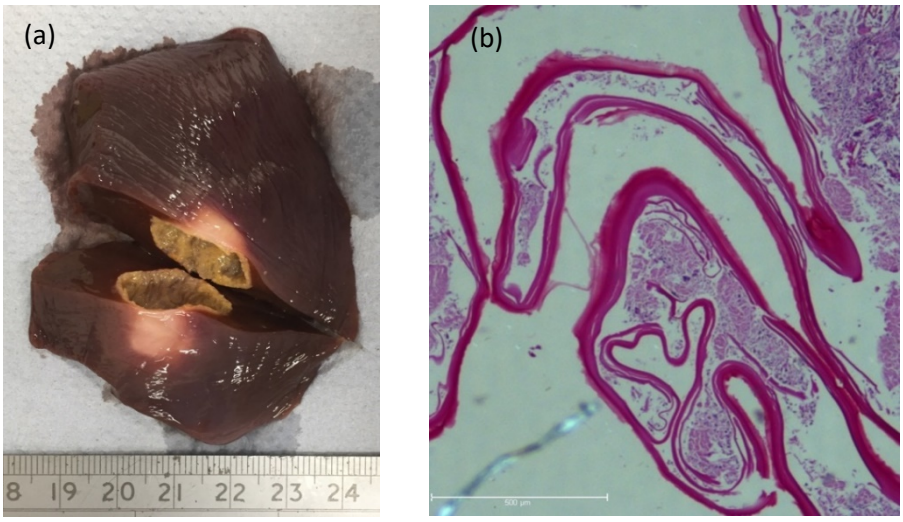

Figure 6-4. Example of an incised unilocular hydatid cyst in bovine liver, identified at abattoir meat hygiene inspection and submitted to the HyData study. Prior to incision, the cyst was filled with clear yellow fluid and no visible protoscoleces, suggesting a sterile cyst. The friable inner laminar membrane is visible and in b) is deeply PAS stained (x4 magnification). E. granulosus was confirmed by PCR and DNA sequencing. The gross image is shown against a cm scale.

There were also 6/77 cystic lesions (6.9%, 95%CI: 1.6-12.2) that had the morphological and/or histological characteristics of *C. tenuicollis* cysts (example Appendix IV-) and 8/87 lesions (9.2%, 95%CI: 3.1-15.3) that were nodular and solid or did not conform to the visual appearance of a cyst.

Histology sections of tissue from the hydatid cysts samples plus one randomly selected control from each sample kit were prepared and stained with PAS and H&E stain. This was undertaken in 154 samples (77 hydatid and 77 controls by MI) due to the late arrival of 10 sample kits, after histopathology was possible. Using the presence of a hyperlaminated acellular laminar layer stained with PAS (Fig. 6-3b and 6-4b and example comparison with H&E, (Appendix IV-d) as an indicator of *E. granulosus*, 11/77 (14.29%, 95%CI: 6.47 – 22.10) were identified as hydatid in origin. A summary of histopathology results against MI is shown in Table 6-5.

Cohen's Kappa statistic to assess the agreement of MI and histopathology beyond chance showed fair agreement between the tests (κ = 0.2278, p<0.0001).

Table 6-5. Histopathology results against meat Inspection. Included are percentage values and Cohen's kappa for level of agreement between tests. Cattle specific results are also included

		Histopathology						
		Cattle			All animals			Cohen's κ
		+ve	-ve	Total	+ve	-ve	Total	
Meat Inspection	+ve	**11** 16.42 100.00	**56** 83.58 44.80	**67** 100.00 49.26	**11** 14.29 100,00	**66** 85.71 46.15	**77** 100.00 50.00	(p<0.0001) **κ = 0.2278** (0.0506) P<0.0001 **Fair**
	-ve	**0** 0.00 0.00	**69** 100.00 55.20	**69** 100.00 50.74	**0** 0.00 0.00	**77** 100.00 53.85	**77** 100.00 50.00	
Total		**11** 8.09 100.00	**125** 91.91 100.00	**136**	11 14.29 100.00	143 92.86 100.00	**154**	

**Percentage values are included in a column/row format*

†*Listed in order: McNemar's p=value (italics), κ value (standard error), κ p=value, level of agreement.*

6.3.4 Summary comparison of agreement between diagnostic tests

A summary of κ outcomes comparing agreement between MI, genus-specific *Echinococcus* spp. rRNA PCR, *genus-specific Echinococcus* spp. cox1 PCR, sanger sequencing of cox1 PCR products and histopathology are shown in Table 6-6.

Table 6-6. Summary of Cohen's Kappa (κ) outcomes for test agreement between MI, Echinococcus spp. genus-specific rRNA and cox1 PCRs, cox1 product DNA sequencing and histopathology.

	Meat hygiene inspection		
Echinococcus rRNA PCR	(p<0.0001) κ 0.2609(0.0408) P<0.0001 Fair	*Echinococcus* rRNA PCR	
Echinococcus cox1 PCR	(p<0.0001) κ 0.3984 (0.0432) P<0.0001 Fair	(p=0.424) κ 0.8128 (0.0556) P<0.0001 Substantial	*Echinococcus* cox1 PCR
Histopathology	(p<0.0001) κ 0.2278 (0.0506) P<0.0001 Fair	(p=1) κ 0.2771 (0.0796) P<0.0001 Fair	(p=0.823) κ 0.3127 (0.0783) P<0.0001 Fair
Listed in order: McNemar's p=value (italics), κ value (standard error), κ p=value, level of agreement.			

6.3.4.1 Evaluation of meat hygiene inspection as a test to identify hydatid cysts

The study aimed to estimate *Se* and *Sp* of meat hygiene inspection as a diagnostic test for hydatidosis in cattle using the samples submitted to the study as a validation subsample of all animals undergoing inspection. The number of hydatid cases detected in cattle and reported via the CCIR system at participating abattoirs during the study was 715. The combined throughput for the 15 abattoirs participating in the study for its duration was 1,048,633 cattle. Based on the above, the apparent prevalence is calculated as 715/1,048,633 = 0.000681 or 0.068%. The corrected Sensitivity (Se_{corr}) and Specificity (Sp_{corr}) were calculated based in the subset of samples assayed by the genus-specific *Echinococcus* spp. cox1 PCR as a gold standard as described by Dohoo *et al.* (2010) as follows:

$$Se_{corr} = \frac{a/_{sf_{T+}}}{a/_{sf_{T+}} + c/_{sf_{T-}}} = \frac{23.1/0.1192}{23.1/0.1192 + 0.1/2.2801} = 30.68\%$$

$$Sp_{corr} = \frac{d/_{sf_{T-}}}{d/_{sf_{T-}} + b/_{sf_{T+}}} = \frac{239.1/2.2801}{239.1/2.2801 + 62.1/2.2801} = 99.48\%$$

where *sfT+* is the fraction (*sf*) of the verified test positives calculated as:

$$sf_{T+} = (a + b)/T_{+} = (85.2)/715 = 0.1192$$

Where T_{+} is the number of positive samples detected at the abattoir and *sfT-* is the fraction of the verified test negatives calculated as:

$$sf_{T-} = (c + d)/T_{-} = 239.1/1{,}048{,}633 = 2.2801$$

Where T_{-} is the number of animals examined at slaughter minus T_{+} ; values for *a, b, c* and *d* are shown in the 2x2 table displaying MI and PCR test results (Table 6-7).

Table 6-7. Meat Inspection Sensitivity and Specificity using a genus-specific Echinococcus spp. cox1 PCR.

		Genus specific *Echinococcus spp*. PCR		
		Positive	Negative	Total
Meat Inspection	Positive	23.1* (*a*)	62.1 (*b*)	85.2 (*a+b*)
	Negative	0.1 (*c*)	239.1 (*d*)	239.2 (*c+d*)
	Total	23.2 (*a+c*)	301.2 (*b+d*)	324.4

**+0.1 was added to all the table elements in order to allow for the calculations to be performed (Haldane-Anscombe correction).*

Because no MI negative samples were found to be positive by PCR, the calculations were performed on alternative values with a modification of the Haldane-Anscombe correction by adding the value of 0.1 to each of the cells of the contingency table (Anscombe, 1956; Haldane, 1940). Based on the overall cattle throughput of participating abattoirs, MI for hydatidosis in cattle has an estimated *Se* of 30.68% (95%CI: 11.91-49.44) and *Sp* 99.48% (95%CI: 99.48-100). However, only overall rather than individual throughput data for participating abattoirs were made available to

the study during its duration, which did not allow the examination of the effect that different inspection teams may have on the results.

6.3.5 Cattle Tracing Records

In total, movement records were obtained for 70/75 (37.6%, 95%CI: 27.3-47.9) cattle sampled in the study. Of the five samples lacking associated movement records, four could not be obtained due to errors in the cattle passport number entered during sampling and one sample arriving after the RADAR request had been made. A total 23/70 samples (32.9%, 95%CI: 21.9-43.9) considered were confirmed as hydatid by PCR and/or histology. A summary table of records for all samples confirmed as hydatid by one or more tests is shown in Table 6-8. There was a highly significant effect of location of travel in cattle testing positive on one or more tests, with 21/23 resident in Wales or an adjacent county (OR=15.47, p<0.0001); 19/23 within Wales (OR=10.13, p<0.0001) and 8/23 within Powys, the area of highest known prevalence in Wales (OR=12, p<0.001); 4/23 cattle with confirmed hydatid cysts had never been to Wales. Their reported transport histories included Herefordshire, Gloucestershire (both adjacent counties to Wales), Staffordshire and North Yorkshire/Humber. In the cases of Staffordshire and North Yorkshire/Humber, this reflects *E. granulosus* identified by molecular methods in two cattle that had never travelled within Wales or a county adjacent to it. For all samples submitted, the mean age of cattle was 94 months (95%CI: 81.5-108.4). Among cattle testing positive for hydatid disease on PCR, DNA sequencing or histopathology, the mean age was 105 months (95%CI: 8.6-132) and for those testing negative on all laboratory tests was 90 months (95%CI: 74.8-105.6). There was no significant effect of sex of the animal (p=0.8366) and a marginally significant effect of breed purpose, with beef cattle more likely to return a positive test result that dairy (OR=2.91, p<0.05).

Table 6-8. Summary data of cattle identified with hydatid lesions at meat hygiene inspection at abattoirs in England and Wales, including test outcomes for Echinococcus spp. rRNA PCR and cox1 PCR; DNA sequencing of cox1 PCR products; histopathology; travel within/outside Wales, region and time spent in region. Age and time spent in region are shown in months.

Site	ID	Breed	Type	Sex	Age	Tissue	cox1 PCR	DNA sequence	Histopathology	Wales	Region	Time spent
0	B	Limousin X	Beef	Female	23	Liver	*Echinococcus spp.*	Poor sequence	Negative	No	Gloucestershire	23
0	E	British Friesan	Dairy	Male	32	Liver	*Echinococcus spp.*	Poor sequence	Negative	Yes	Dyfed	32
0	G	Charolais X	Beef	Male	35	Liver	*Echinococcus spp.*	*E. granulosus*	Negative	Yes	Powys	35
5	B	Hereford X	Beef	Female	147	Liver	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Dyfed	120
5	C	Aberdeen Angus X	Beef	Female	194	Lung	*Echinococcus spp.*	*E. granulosus*	Negative	Yes	Powys	75
9	A	Limousin X	Beef	Female	108	Liver	*Echinococcus spp.*	Poor sequence	*E. granulosus*	Yes	Powys	108
9	L	British Friesan	Dairy	Female	156	Liver	Not tested	Not tested	*E. granulosus*	Yes	Dyfed	156
10	B	Holstein Fresian	Dairy	Female	183	Lung	*Echinococcus spp.*	Poor sequence	Negative	Yes	Dyfed	183
10	E	Holstein Fresian	Dairy	Female	195	Liver	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Powys	134
10	G	Charolais X	Beef	Female	180	Liver	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Dyfed	180
10	H	Aberdeen Angus X	Beef	Female	180	Lung	*Echinococcus spp.*	*E. granulosus*	Negative	Yes	Dyfed	180
10	I	British Friesan	Dairy	Female	39	Lung	*Echinococcus spp.*	*E. granulosus*	Negative	Yes	Dyfed	39
12	D	Limousin X	Beef	Female	168	Lung	*Echinococcus spp.*	*E. granulosus*	Negative	Yes	Powys	168
12	E	Holstein Fresian	Dairy	Female	73	Liver	*Echinococcus spp.*	*E. granulosus*	Negative	No	North Yorks/Humber	72
12	K	Holstein Fresian	Dairy	Female	141	Lung	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Dyfed	141
12	M	Holstein Fresian	Dairy	Female	67	Lung	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Dyfed	67
12	N	Limousin	Beef	Female	99	Lung	*Echinococcus spp.*	*E. granulosus*	Negative	Yes	Powys	65
12	P	Aberdeen Angus	Beef	Female	125	Liver	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Dyfed	125
13	A	Hereford X	Beef	Female	106	Liver	*Echinococcus spp.*	E. granulosus	*E. granulosus*	Yes	Dyfed	106
13	D	British Blue X	Beef	Male	15	Liver	*Echinococcus spp.*	*E. granulosus*	Negative	No	Staffordshire	15
13	E	Simmental	Beef	Female	194	Liver	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Powys	192
13	G	Holstein Fresian	Dairy	Female	52	Lung	*Echinococcus spp.*	*E. granulosus*	*E. granulosus*	Yes	Clwyd	52
14	D	Limousin X	Beef	Female	23	Liver	*Echinococcus spp.*	*E. granulosus*	Negative	No	Herefordshire	23

6.4 Discussion

6.4.1 Tracing movements of affected cattle

Using molecular techniques and histopathology in cattle as a sentinel species, this study has confirmed for the first time the presence of *E. granulosus* in England and outside of known areas of endemicity. The study identified four cases that have not travelled within known prevalence hotspots in Wales. Two cases were resident in counties adjacent to Wales; one in Herefordshire, where historically, pockets of human infection have been reported (Craig *et al.*, 1996; Palmer & Biffin, 1987); a second in Gloucestershire, were a reported foxhound worker was believed to have contracted the disease (Craig *et al.*, 2012). *E. granulosus* transmission in areas adjacent to those in Wales, where no control programmes have been carried out, has been suggested as a potential route of re-infection into Wales, following control efforts in the country (Public Health England, 2014). To the author's knowledge, the two other cases in Staffordshire and North Yorkshire/Humber are the first to be confirmed by molecular methods in these regions. Testing has identified travel within Powys, Wales overall and adjacent counties to Wales as highly significant risk factors for *E. granulosus* infection in cattle. These findings support the existing evidence identifying these as areas of high prevalence within the UK (reviewed in Chapter 1) and give confidence in the quality of the data produced.

A survey by Temple *et al.* (2013) of slaughter records from Wales identified 757 reported hydatidosis cases in 893,379 cattle slaughtered at 19 abattoirs between 2010-2011, with movement records traced to find the most likely locations of infection (Temple, Jones and Brouwer, 2013). The study demonstrated that *E. granulosus*, based on MI identification, was not confined to mid-Wales and surrounding regions, and reported a wide distribution throughout Wales, England and some locations in Scotland. Estimated prevalence per 100,000 cattle slaughtered by postcode area confirmed a higher prevalence in in mid-Wales and Herefordshire, but also the Midlands, Aberdeenshire and Perthshire in Scotland, and a small pocket to the East of Manchester. Through the use of molecular methods and histopathology, the HyData study builds on this evidence of a wider national

distribution of *E. granulosus* by providing molecular and histological evidence of the parasite as the causative agent. All cases confirmed as *E. granulosus* by the study were found in regions where Temple *et al.* (2013) had reported hydatid cases. Together, these studies demonstrate the value of using abattoir disease records and movement data for mapping hydatid disease and how abattoir sampling, histology, species-specific PCR and sequencing techniques can advance and strengthen the evidence of a wider disease distribution in the UK.

6.4.2 Evaluation of diagnostic *Se* and *Sp* of meat inspection for cystic hydatidosis in cattle at slaughter

The study has produced preliminary estimates of the *Se* and *Sp* of MI as a diagnostic test but due to the lack of individual site baseline throughput information, it has not been able to complete the calculations optimally. Based on the total throughput for participating abattoirs in the HyData study, MI detection for hydatid lesions in cattle had an estimated diagnostic *Se* of 30.68% (95%CI: 11.91-49.44) and *Sp* 99.48% (95%CI: 99.48-100). These findings are in broad agreement with those of Wilson *et al.* (2019), who reported a diagnostic *Se* of 24.9% and *Sp* of 98.9% in their MI evaluation study of hydatid detection in cattle using the gold standard reference test (Wilson *et al.*, 2019). The MI process has been challenged in recent years for its low sensitivity for detection of important public health hazards (Stärk *et al.*, 2014). Studies have reported a low sensitivity in MI for diagnosis of parasitic lesions in pigs using Latent Class Analysis (Bonde *et al.*, 2010) and variability in the inspection process between abattoirs (Schemann *et al.*, 2010).

The reference standard for detection of hydatid cysts in intermediate host organs involves palpation of the organ, typically the liver or lungs, cutting 5-6mm slices throughout the organ and lesions, and examination, including histopathology for identification of a germinal membrane (Banks *et al.*, 2012; Eckert *et al.*, 2002; Lloyd *et al.*, 1998; Thompson, 2017). However, it is unsuitable to perform in a high-speed processing environment and leads to damage of liver tissue, rendering it unfit for commercial sale (Wilson *et al.*, 2019). Logistically, it was not possible to use the reference standard test in the thesis, as the study undertook simultaneous sample

collection at multiple abattoirs around the country. It would also not have been possible with the strict health and safety regulations for visitors at food production units (BMPA, 2014). Evaluation of MI using alternative methods, such as Latent Class Models, that do not rely on a gold standard reference test, were considered (Dohoo, 2010; Hartnack *et al.*, 2013). However, there were several limitations to this approach in this setting; firstly, an assumption of such a test would be violated, in that the *Se* and *Sp* of the selected diagnostic tests e.g. MI and PCR would not be constant across subpopulations with different prevalence i.e. different abattoirs or regions; secondly, the necessary baseline throughput data for individual abattoirs were not made available to the study; thirdly, participating abattoirs only sent a small proportion of the hydatid samples recorded by CCIR during the study period and they did not represent a random sample of those recorded.

Prevalence of hydatid disease in livestock is age dependent, and as such, surveillance studies at slaughter should be stratified for age (OIE, 2018). The sensitivity of hydatid diagnosis based on MI is quite low in early infections (e.g. in younger animals) (Craig *et al.*, 2015). Early hydatid lesions can appear as small white nodules, which may be missed during inspection (Liu, Che, & Chang, 1993). In order to maximise sample return, this study selected the highest throughput abattoirs based on cattle over 8 months of age, which is likely to have affected the evaluation outcome of MI *Se* and *Sp* as a whole. Further studies, reflecting cattle of all ages and stratified accordingly would be required. Equally, clustering at the abattoir and inspector level would need to be taken into consideration.

The specificity of MI may also be affected by other conditions or infections with similar lesions to hydatid disease (Craig *et al.*, 2015). The high estimated *Sp* for MI in this study suggests the impact of misclassification of cysts in cattle from other causes, such as liver fluke, granulomas and congenital cysts (Eckert *et al.*, 2002) would be low. In contrast, although sheep were not the sentinel species used for this study, the small number of samples submitted from sheep as hydatid, all from one abattoir, were largely misclassified and were confirmed by PCR, DNA sequencing and histology as *C. tenuicollis*, the larval stage of *T. hydatigena.* Misclassification of *C. tenuicollis* cysts in particular are thought to affect the specificity of MI for hydatid cysts in sheep

(Craig *et al.*, 2015). The reason why these misclassified samples were all submitted from one abattoir is unclear, nor can it be assumed that this reflects the degree of misclassification of hydatid lesions in sheep across different abattoirs, however this is an interesting finding and supports the rationale for further use of confirmatory testing methods in the validation process of MI.

The accuracy of MI can be improved by additional methods, such as histopathology, immunohistopathology and PCR testing of suspect lesions (Eckert *et al.*, 2002). Molecular methods, such as PCR and DNA typing have been shown to be useful for further identification of cysts discovered at slaughter (Bardonnet *et al.*, 2003; Boufana *et al.*, 2014; Guo *et al.*, 2019; Siles-Lucas *et al.*, 2017), although to the author's knowledge, this is the first study to attempt evaluation of the process using a molecular method as a reference standard. The study reported 'fair' agreement between MI, PCR and histopathology detection methods for hydatid lesions. The PCR and histology methods rely on representative sampling of the lesion submitted and in the case of PCR, the quality and quantity parasite DNA present in the sample. Many cysts had been incised as part of the inspection process prior to sample submission, affecting the amount of parasite material, cyst content and lesion architecture available for analysis. Improvements in sampling, for example by stipulating submission of intact cysts wherever possible, could improve the downstream molecular and histological diagnosis of submitted lesions. A more objective assessment of the effect of time between sampling and laboratory testing on the quality of the sample available would also help to assess if this was a contributing factor to test outcome and the level of agreement between MI and laboratory tests. The current level of 'fair' agreement must be taken into account when interpreting the estimation of MI *Se* and *Sp* as a validation exercise and would need to be improved if this approach is to be used to evaluate MI for CE and other diseases identified at slaughter.

While the estimated *Se* and *Sp* values must take into account the limitations of the evaluation approach here, it is hoped that obtaining the true baseline data for the abattoir in the study, a continued aim of this work, will provide a more robust evaluation of MI in the identification of hydatid cysts. The value of such research

outputs to the meat hygiene and farming industry cannot be underestimated. The main aim of MI is to remove meat products unsafe or unfit for human consumption, and with respect to hydatid disease, the correct identification of lesions in this respect appears to be very high. However, the estimated low sensitivity of the process could mean that the true prevalence of hydatid disease in cattle (and possibly sheep also) is being underestimated, limiting effective disease condition reporting to farmers via the CCIR system. From a disease control perspective, it is important that hydatid lesions identified in livestock at slaughter are condemned and disposed of correctly (Craig *et al.*, 2015). On a wider scale, using hydatid disease as a pilot, the importance of evaluating the MI process for other diseases, especially those with a direct risk to human health, animal health and a large economic impact on the livestock industry, is clear.

6.4.3 Study design and participation

In order to sample hydatid lesions at several abattoirs in England and Wales, the study developed a sampling kit for MHIs and OVs to return by post. Existing surveillance programmes that involve a system of collection of samples at MI with postal return to a reference laboratory include notifiable diseases under official control, such as serum sampling of breeding boars for Aujesky's disease, non-notifiable diseases requiring surveillance under EU law, such as *Trichinella spiralis* and surveillance conducted by the FSA for *Campylobacter* in broiler chickens (FSA, 2018d). Sampling protocols for such schemes are explicitly described in the Manual of Official Control for all FSA (FSA, 2018d).

The majority of participating abattoirs were located in England, with only one in Wales. It is possible that cattle originating from Wales, the area with the highest reported prevalence of *E. granulosus* (Buishi *et al*., 2005; Lloyd *et al*., 1998; Mastin *et al*., 2011; Palmer *et al.*, 1996c) is underrepresented in the study. Large throughput abattoirs were selected to obtain samples from livestock transported long distances across the country to the sites. However, as no additional information was collected about the control samples obtained (as it would not have been practical to do so

during sample collection), it is not possible to evaluate how representative the overall hydatid sample set is within the wider cattle population.

This study received 87 samples identified as hydatid on routine meat hygiene inspection, plus 257 control samples from 10 abattoirs in England and Wales. A further 4 participating abattoirs did not submit samples to the study. This total number of samples represents a small proportion (12.3%) of the hydatid cases reported on the CCIR system for these abattoirs during the study period. The study required that *all* lesions identified as hydatid on inspection be sampled and submitted. Due to the commercially sensitive nature of the data, it was not possible to obtain throughput data for the individual abattoirs for the duration of the study. These missing data and the low rate of sample return have reduced the value of applying inferential statistics to estimate the sensitivity and specificity of the meat hygiene inspection process using hydatid disease as an exemplar. This must also be taken into consideration when interpreting the findings relating to geographical distribution of infection.

6.4.4 Conclusions

Despite the limitations outlined, the abattoir study has reported important findings. Firstly, cases of hydatid disease identified at MI, and for the first time confirmed as *E. granulosus* by molecular methods in the UK, occurring outside of known areas of endemicity. Further confirmatory testing of abattoir samples, combined with accurate throughput data, would allow prevalence within new identified areas of infection to be explored. A better understanding of the geographical distribution of disease, strengthened by species-specific PCR testing, could allow targeted sampling of farm dogs and hunting packs based on trace-back of livestock hydatid slaughter data (Lett *et al.*, 2018). Secondly, as a proof of concept, confirmatory PCR and histology have been used to evaluate MI for hydatid disease in cattle. Estimates for *Se* and *Sp* based on available data suggest a low sensitivity and high specificity for MI hydatid detection, in agreement with research findings for post-mortem MI as a whole and specifically with hydatid disease. The low sensitivity of inspection is likely to result in underestimation of the true prevalence of hydatid disease in the cattle population. This could limit the value of disease reporting via CCIR, reducing the quantity and quality of information returned to farmers and underestimating the overall burden of disease in livestock. The high specificity of inspection suggests low misclassification and lower economic loss through product wastage. If improvements can be made to optimise agreement between PCR methods, histology and MI hydatid detection, the study proposes the potential use of this methodology to validate abattoir data prior to use in surveillance and reporting of hydatid disease. The next step for this work is to obtain the individual throughput data for participating abattoirs, to better inform the evaluation and validation process piloted here. The study has highlighted important logistical issues with data collection, compliance in collection and laboratory testing that must be addressed if this pilot work is to be expanded to wider use. A 2-stage validation approach relies on both representative data from the first stage at MI inspection and the validation subsample at the second stage. If such limitations are addressed, this methodology has the potential to report robust data that reflects both the MI process and the burden of hydatid disease in the wider UK livestock population.

Chapter Seven

Concluding Discussion

7. Concluding discussion

7.1. Overview

Despite a long history of *E. granulosus* and *E. equinus* transmission in the UK, there is a paucity of recent research, particularly investigating *Echinococcus* at the species level in known endemic regions and possible new areas of transmission. This thesis attempts to bridge the gap in research where molecular studies applied to at risk animal populations at the national level are needed. Together, the four thesis studies have addressed three broad aims; firstly, to investigate the hypothesis that *E. granulosus* in the UK is not confined to traditionally known areas of high prevalence. Secondly, to investigate *Echinococcus spp*. in canine populations and livestock at slaughter and build on the current understanding of the UK burden and distribution of infection; thirdly, to gather important data on known and potential risk factors of *Echinococcus spp.* infection in definitive canine hosts in the UK. A fourth aim emerged during the abattoir study planning; to validate the meat hygiene inspection process for hydatid disease in livestock using molecular tests and histopathology as the gold standard as a proof of concept exercise.

This chapter brings together the key findings from the four studies relating to the overarching aims of the project, the shared strengths and limitations of the research and the conclusions and recommendations of the work. This is explained by revisiting the themes and aims described in the introduction that underpin the rationale for this research. A fourth area of research that developed during the abattoir study is also discussed.

7.2. Key themes

7.2.1. Distribution of *E. granulosus* in the UK

The four thesis studies report evidence that *E. granulosus* distribution extends far beyond the known areas of high prevalence in the UK. Results from the abattoir and farm dog studies also suggest that Wales remains a focus of *E. granulosus* transmission.

A summary of UK regional and country locations where the HyData studies have identified *Echinococcus* spp. in at-risk animal populations (reported at a level that maintains participant anonymity) is illustrated in Table 7-1.

Table 7-1. Summary of positive Echinococcus spp. laboratory test locations for the four HyData studies by coproELISA ●, Genus-specific coproPCR ▲, E. granulosus G1 coproPCR ▲, E. granulosus coproPCR + DNA sequencing ▲, E. equinus coproPCR ▲ and histopathology ■ Regional results in England for the zoo study are not shown to avoid site identifiers.

Region	Farm	Zoo	Hunt	Abattoir
Wales	●▲	NA		●▲▲■
Scotland	●▲		●	NA
N. Ireland	▲	NA		NA
England	●▲	●▲		▲▲
North East	●▲			
North West	●		●▲	
Yorkshire/Humber				▲▲
East Midlands				
West Midlands				▲▲
East Anglia	●			
South East				
South West	●		●	▲

One foxhound pack in the North of England tested positive on coproPCR for *E. granulosus* and three foxhound packs tested positive for *Echinococcus spp*. coproantigen in the South West of England, the North of England and the Scottish Borders. To the author's knowledge, this represents the first reported cases of *E. granulosus* and Echinococcus *spp.* coproantigen results in hunting packs in these regions of the UK. Together with a recent report of two hunts in Northumberland confirmed positive for *E. granulosus* (Lett *et al.*, 2018), this supports a picture of *Echinococcus spp*., and importantly zoonotic *E. granulosus* transmission in hunting packs within novel regions of the UK. Five hunting packs located in Wales took part in the HyData study and none tested positive for *Echinococcus spp* coproantigen or

coproDNA. In contrast, Lett et al (2018) identified five Welsh hunts positive for *Echinococcus spp.* coproantigen and/or *E. granulosus* coproDNA, of which two also tested positive for *E. equinus* coproDNA (Lett *et al.*, 2018).

E. granulosus was identified in farm dogs from five farms located in Wales, the North of England, Scotland and Northern Ireland. At the time of writing, Ireland is believed to be non-endemic for *E. granulosus*, with no autochthonous human cases of cystic echinococcosis reported to date (Torgerson and Budke, 2003; Deplazes *et al.*, 2017). The finding of a farm dog testing positive for *E. granulosus* on coproPCR in Ireland calls for further information gathering and sampling of dogs on this and surrounding farms, in particular noting any travel history of dogs to known endemic areas. A priority remains to confirm the result with sequencing of the PCR product, which unfortunately was not possible within the time of the study. Given that Ireland is declared free of *E. granulosus*, discussion is needed with the competent authorities on the best course of action, while maintaining the anonymity of NSA member details unless consent is given from the farmer for these details to be made available as part of further investigation.

The farm study also identified 8 farms with dogs positive for *Echinococcus spp*. coproantigen; two in the North East of England were also confirmed as *E. granulosus* on coproPCR. These farms reflect a wide distribution within England, Wales and Scotland. This study found a significant association between a positive result on either coprological *Echinococcus* spp. test conducted and farms in the NSA Wales member region, supporting a picture of re-emergent *Echinococcus* spp. transmission in that region (Buishi *et al*., 2005; Deplazes *et al*., 2017; Mastin *et al.*, 2011). The farm dog study confirmed three positive farms in Wales, two for *E. granulosus* DNA and one for *Echinococcus spp.* coproantigen. Two farms at the centre of a known endemic area of Powys (Buishi *et al.*, 2005; Mastin *et al.*, 2011) and one in the North of Wales. Historically, the foci of human and animal *E. granulosus* infections in Wales have been Powys in mid-Wales and Glamorgan in the South East (Palmer *et al.*, 1996; Buishi *et al.*, 2005; Mastin *et al.*, 2011). A report of *E. granulosus* in a foxhound pack in Clwyd, in the North of Wales (Stallbaumer, 1987) and the finding in this study of *E. granulosus* in a farm in the adjacent region of Gwynedd suggests that the North of

Wales is a possible site of existing or re-emergent infection that merits further investigation.

The zoo project identified five zoos in England with enclosures testing positive for *Echinococcus spp*. coproantigen and with one European Wolf enclosure testing positive for *E. equinus* DNA. To the author's knowledge, these are the first reports of *Echinococcus spp*. in captive canids in UK zoos. Although not considered a zoonotic pathogen, fatal cases of *E. equinus* have been reported in intermediate host species in UK zoos (Boufana *et al.*, 2012; Denk *et al.*, 2016). The role of competent canid hosts as an *Echinococcus* spp. transmission risk to other enclosures within the same zoo environment had not been explored in the UK. Evidence that *Echinococcus* spp. and namely *E. equinus* is present in UK zoo canids, and evidence of feeding raw offal from sheep and cattle shows the potential for zoonotic *E. granulosus* transmission is present in this setting. This presents a putative zoonotic transmission risk to personnel working closely with zoo canids and calls for further research to establish potential routes of *E. equinus* (and *E. granulosus*) transmission.

The non-probability sampling design of the canine studies, low study numbers and likely resultant selection bias mean that prevalence estimates of infection would not have been a suitable outcome of the data from these studies. However, the studies have indicated, at the species level, that *E. granulosus* is cycling within UK farm dog and hunting hound populations and *E. equinus* is cycling within zoo canids. These findings, together with widely distributed positive cases of *Echinococcus* spp. coproantigen point to areas where surveillance studies designed to estimate prevalence of infection, most importantly with zoonotic *E. granulosus* are needed.

Through the matching of hydatid cases in cattle to movement records via the CTS, the abattoir study was able to determine whether cattle infected with *E. granulosus* had ever travelled to regions of known endemicity. While the majority of cattle confirmed positive had originated or spent extended periods of time in endemic regions in Wales, the study also identified five cattle that had never travelled within Wales. Two of these cattle originated form areas adjacent to prevalent hotpots, though three were from regions further afield. The findings support pilot study data

mapping the distribution of cattle hydatid cases identified at meat inspection, which found widespread cases throughout Wales, England and some in Scotland (Temple *et al.*, 2013).

Cattle identified with hydatid lesions on inspection that had originated in or travelled in Wales were significantly more likely to have hydatid disease confirmed by PCR, DNA sequencing or histopathology ($p<0.0001$), supporting both definitive host data indicating that Wales remains an endemic and likely re-emergent hotspot for *E. granulosus* (Buishi *et al.*, 2005; Mastin *et al.*, 2011). This study supports the findings of Temple at al. that also reported cattle throughout Wales testing positive for hydatid disease at inspection (Temple, Jones and Brouwer, 2013). Here, we also confirm the identity of the cysts in these cattle as *E. granulosus* on both tissue PCR and DNA sequencing. Phylogenetic analysis of the *E. granulosus* PCR product sequence data fell beyond the scope of this project; however, this is an important next step to build on this work. Haplotype network analysis of hydatid PCR sequence data is increasingly used to explore the phylogenetic relationships within and between *Echinococcus* host species (Boufana *et al.*, 2015; Dán *et al.*, 2018), revealing valuable information on transmission dynamics. The study confirms cattle as a valuable sentinel species for distributions studies of *E. granulosus*. Similar studies have successfully used molecular techniques to explore the epidemiology of CE in livestock (Andresiuk *et al.*, 2013), but to the author's knowledge, this is the first to do so in the UK.

7.2.2. Risk factors for *Echinococcus* spp. transmission in UK animal study populations

The three canine projects in the study have identified risk factors for *Echinococcus* spp. transmission within the husbandry and practices of their respective settings, including possible infractions of the law in the feeding of ABP and sub-optimal applications of disease prevention and control guidelines for echinococcosis.

The value of the thesis was not only in contributing to a better understanding of *E. granulosus* distribution in the UK, but also in identifying possible risk factors for

infection that could be further explored and targeted in disease control interventions. The risk factors in question were not necessarily always associated with positive test results, possibly due to the small sample size resulting in low statistical power. Nevertheless, the questionnaire responses identified that such risk factors exist in UK farms, hunting packs and zoos, and as a consequence, there was the potential for *Echinococcus* spp, and importantly zoonotic *E. granulosus,* to complete its lifecycle in these settings.

The main risk factors that were explored were a) the feeding or scavenging or raw carcases, in particular offal; b) sub-optimal worming and c) collection and disposal of canine faeces. All these risk factors are associated with management practices that could be targeted as part of an integrated approach to *Echinococcus* spp. control.

7.2.2.1. Raw food feeding

A common feature of all three canine study populations was the feeding and/or scavenging of raw meat and offal material from sheep, cattle and (in zoo and hunt studies) horses. As discussed in chapters 1, 3 and 4 , in the case of hunting packs and zoo canids, infrastructure and legislation exist to allow hunting packs and zoos to source ABP from the slaughter industry and as fallen stock (DEFRA, 2011a, 2011b; The European Parliament and The Council of European Union, 2009). In the case of farm dogs and hunting hounds, there is opportunity for the scavenging of carcases on farm land (Buishi *et al.*, 2005; Harris & Dorning, 2017; Mastin *et al.*, 2011; Otero-Abad & Torgerson, 2013).

Over one third of participating farms (36.2%) in the HyData study reported purposefully feeding raw meat and offal from fallen stock to their dogs, with 8.5% specifically reporting feeding offal from fallen sheep and cattle, the highest risk material for *E. granulosus* transmission. Intact fallen stock is classed as Category 1 ABP material until it is collected and processed by approved premises into Category 1 and Category 2 ABP for disposal or further permitted uses. Unless a farm is registered as an approved kennel end user of Category 2 ABP material, the feeding of fallen stock to farm dogs contravenes current EU legislation on the use of ABP

material (DEFRA, 2011b). The questionnaire did not specifically ask whether farmers slaughtered animals on-site for personal consumption, so access to raw meat and offal from this practice cannot be excluded. However, the findings reported relate to questionnaire responses specifically indicating material sourced from fallen stock. The feeding of food scraps of offal has been associated with a significantly higher risk of *Echinococcus* spp. coproantigen positivity (OR=1.84) and home slaughter of sheep (OR= 2.49) in Welsh farm dogs (Buishi *et al.*, 2005) though access to fallen stock was not investigated. As a transmission risk of zoonotic *E. granulosus*, the feeding of raw meat and offal from fallen stock is a practice that needs to be brought to the attention of relevant stakeholders, including DEFRA, DAERA, the NSA, farm assurance schemes and sheep farmers, with the urgent advice that it is stopped. This practice presents a known risk of zoonotic *E. granulosus* transmission. Current advice to farmers from the NSA and the Red tractor Farm Assurance Scheme includes avoiding access to fallen stock carcases and raw meat and offal (NSA, 2019b; RTA, 2013). Efforts to stop this practice could be made through a renewed and targeted campaign of farmer education and by making avoiding such practices, alongside adequate risk-based worming of farm dogs, a condition of NSA and farm assurance scheme membership with regular review. The NSA do not currently gather information on whether members keep dogs on-farm (N. Noble, NSA, pers. comms.), and doing so, alongside information flock holdings could help targeted health education.

Studies of echinococcosis in UK farm dogs have investigated and identified roaming as a significant risk factor for *Echinococcus* spp. infection in farm dogs, as an indicator of possible access to scavenging fallen stock (Buishi *et al*., 2005; Mastin *et al.*, 2011). However, evidence of witnessed scavenging of carcases by farm dogs has been lacking. In the HyData study, over three-quarters, 36/47 (76.6%), of participating farms indicated access of farm dogs to land shared with livestock and almost half (44.7%) of farms reported witnessing farm dogs frequently or occasionally scavenging carcases of fallen stock. Witnessed scavenging of ABP by dogs has been reported in studies of *E. granulosus* in stray dogs scavenging waste from urban abattoirs in Nairobi, reporting 72% infection with *E. granulosus* in stray dogs tested

(Wachira, Sattran, Zeyhle, & Njenga, 1994), and Peru, reporting 6.25% infection with *E. granulosus* in stray dogs tested (Moro *et al*., 2004). To the author's knowledge, the thesis gives the first report of witnessed carcase scavenging by farm dogs in the UK and indicates an important transmission risk is present. The onus is on farmers to prevent access of farm dogs to carcases on land by preventing free roaming behaviour and ensuring that regular checks are made to locate and collect dead livestock on farm land. The importance of this should be made clear in herd and flock health programmes, advice issued through NSA on disease control and as part of Farm Assurance Schemes.

Foxhound packs in the HyData study were significantly more likely to feed raw ABP in all the risk categories for *Echinococcus* spp. infection, including very high risk for *E. granulosus* and *E. equinus* transmission than other hunt types. Beagle packs were significantly more likely to feed high risk ABP for *Echinococcus spp.* infection. Domestic dogs fed raw offal are more likely to test positive for *Echinococcus* spp. coproantigen (Buishi *et al.*, 2006; Moro & Schantz, 2009), and the feeding of infected offal perpetuates *Echinococcus* spp. transmission (Eckert & Deplazes, 2004; Otero-Abad & Torgerson, 2013). The derogation to the law that permits certain types of category 2 ABP to be fed to hunting hounds in the UK stipulates that this must not include offal (DEFRA, 2011b). In the HyData study, 23 out of 32 hunts (71.9%) reported feeding raw ABP (meat and offal) from sheep and cattle fallen stock, including 14 out of 32 reporting feeding offal/viscera (43.8%). Lett *et al.* (2018) reported 8 out of 16 hunts (50%) in England and Wales feeding livestock carcases/raw offal to hounds (Lett *et al.*, 2018). These studies together suggest that the feeding of raw livestock meat and offal remains a current and common practice in UK hunts, presenting an important risk factor for *E. granulosus* transmission in many parts of the country. This practice should be raised and discussed with DEFRA, the Hunting Office, the NSFCo and hunts through their respective hunt associations, in particular the Masters of Foxhounds Association and the Association of Masters of Harriers and Beagles. There is scope to underscore the risks of this practice in the Code of Practice for the Welfare of Hounds in Hunting Kennels (CHA, 2015) and during CHA hunt kennel inspections, include evidence that offal from fallen stock is

being separated and discarded appropriately in the list of controls. A risk may remain from hounds scavenging fallen stock on farmland, which underpins the point made earlier highlighting the importance of farmers regularly recovering fallen stock. Hunts could be encouraged to report sightings of fallen stock to farmers during the long distances covered in a day's hunt.

In the HyData zoo study, half of participating zoos in study fed raw ABP from fallen stock to canid enclosures, with 7 out of 20 (35.0%) feeding carcases and viscera from sheep and cattle fallen stock. The majority of fallen stock ABP fed to zoo canids was of equine origin, with 16 out of 20 (80%) feeding raw equine ABP. These practices represent transmission risks for both *E. granulosus,* suggesting a public health and animal health risk, and *E. equinus,* suggesting a potential infection risk to intermediate host species sharing the zoo environment, supported by the finding of a positive wolf enclosure testing positive for *E. equinus* in this study. The zoo study included 14 canid and hyaenid species; across participating zoos, there were wide variations in the diets fed to this variety of species, with no standard diet fed for one particular species group. This may affect the level of risk of *Echinococcus* spp. infection through dietary choices at individual zoos, and as such, worming protocols should be risk-based on a case-by-case, rather than species basis. UK zoos are subject to Government licensing inspections and membership requirements of BIAZA, and although both include provision of preventative healthcare and dietary needs, there are no specific controls to mitigate the risk of *Echinococcus* spp transmission through the feeding of high-risk ABP. There is scope for knowledge exchange with the competent authorities regulating zoo licensing, BIAZA and individual zoos to consider permitted feeding practices in the context of the zoonotic disease risk they pose alongside meeting nutritional and behavioural needs.

Across all the canid studies, it was evident that the storage methods of ABP and raw food products would not be likely to mitigate the risk of *Echinococcus* spp. transmission. Integral to the advice on raw food feeding to all stakeholders should be advice on cold storage (a minimum of one week frozen at -17˚C to -20˚C in a typical household freezer (ESCCAP, 2017)) or cooking (internal temperature of minimum 65˚C for 10 minutes (ESCCAP, 2017; CHA, 2015; Torgerson, 2014)) of ABP. However,

optimally, the feeding of raw offal from livestock and horses should be avoided as a practice entirely in all three study settings.

7.2.2.2. Worming

Removal or reduction in the *E. granulosus* burden in definitive canine hosts will have the greatest and quickest effect to reduce active transmission through rapid decrease in egg production and resultant infection pressure to livestock (Craig *et al.*, 2017). All three canine study populations are recommended to be regularly wormed as part of existing best management practice guidelines (BIAZA, 2019; NSA, 2019; CHA, 2015), and in the case of zoos, as part of Government licensing (Great Britain., 1981). Risk-based worming recommendations for canines fed raw meat/offal or able to roam and potentially scavenge carcase material are to worm with a praziquantel-containing wormer every 6 weeks to prevent *E. granulosus* egg shedding (ESCCAP, 2017). However, worming a minimum of 4 times annually will reduce reinfection (Craig *et al.*, 2017; Eckert *et al.*, 2002; Vuitton *et al.*, 2017). Worming practices across the three canine studies were generally suboptimal for the prevention of *Echinococcus* spp. infection in both selection of wormers used and frequency of administration.

The farm study encouragingly reported that 93.6% of farms in this study regularly wormed their dogs and of these 95.2% were doing so with a product containing praziquantel. However, the finding that only 57.5% of farms using praziquantel were doing so at a minimum 4 times per year, the minimum for non-risk assessed dogs, and only 7.1% at 6-weekly intervals, the recommended amount for this risk group, is an important finding. The study also reported a significant association between suboptimal worming with praziquantel (less than 4 times per year or not according to manufacturer instructions) and a positive result on *E. granulosus* G1 coproPCR. This agrees with the findings of Buishi *et al.* (2005) in a study of echinococcosis in Welsh farm dogs, where infrequent worming (<4-month intervals) was a significant risk factor for positive coproantigen results (Buishi *et al*., 2005). Together with the evidence of re-emergence of *E. granulosus* in areas of Wales where a worming-based hydatid control scheme had prematurely ended (Buishi *et al*., 2005; Craig *et al.*, 2017;

Mastin *et al.*, 2011), these findings highlight the importance of risk-based praziquantel dosing in the sustained control of *E. granulosus* in farm dogs. As with advice on risks of raw meat/offal feeding, optimal risk-based worming advice should be issued through a renewed and targeted campaign of farmer education and by making optimal praziquantel worming a condition of NSA and farm assurance scheme membership with regular review. Furthermore, as over three-quarters (76.6%) of farms reported seeking worming advice from their veterinary surgeon, it is important that vets are well informed on the risks of *E. granulosus* transmission on-farm and the best worming products available. This is particularly important at a time when many competing endectocidal products are available on the market and selection based on optimal parasite coverage can be complex.

All hunts taking part in the study reported regularly worming their hounds, although 44.3% were not using a praziquantel-based wormer. This is a lower proportion than the 56% of hunts reported as not using praziquantel in a survey of 16 foxhound packs in England and Wales (Craig *et al.*, 2012; Lett, 2013). Of the 66.7% of hunts using praziquantel, only a quarter were doing so at a minimum of 4 times yearly and none 6-weekly. Current recommendations in the Code of Practice for the Welfare of Hunting Hounds, to worm with a praziquantel wormer twice yearly, at the start and end of the hunting season (CHA, 2015), need to be updated to a minimum of 4 times yearly or ideally 6-weekly if fed raw ABP, although the logistics and cost of doing so may preclude the latter. There is clear scope to inform hunt on risk-based worming of hounds, ideally by making it a requisite of hound health programmes overseen by the CHA. However, knowledge exchange with the Hunting Office, researchers, ESCCAP, hunts and vets is needed to establish worming protocols that are practical, effective, economical and sustainable in packs of hounds. Furthermore, hunts need to be strongly advised against the practice of empirically administering livestock and equine worming products to their hounds; a practice that contravenes licensing laws, carries potential adverse health consequences for the hounds and is likely to result in ineffective worming for *Echinococcus* and other parasites. Research is needed to assess the risks associated with the practice of puppy walking in hunts, where

members of the public care for young hounds for extended periods and may be placed at risk of zoonotic *E. granulosus* transmission.

Due to the lack of licensed products for captive wildlife species, zoos likely worm canids based on extrapolated protocols for domestic dogs, using veterinary products under the prescribing cascade (VMD, 2015). Worming of zoo canids is stipulated in legislative and guideline documents applicable to UK zoos and wildlife parks (BIAZA, 2014, 2019; Council of the European Union, 1992, 1999; DEFRA, 2012b; EAZA, 2014; Great Britain., 1981; Health and Safety Executive., 2012), although none specify wormer type or frequency of administration. In 22 participating zoos, 60% reported regular worming of canid enclosures, with 50% using a praziquantel-based wormer and only 8% of zoos doing so a minimum or 4 times annually for canid species. No zoos were using a 6-weekly worming protocol for the optimal prevention of *Echinococcus* spp. transmission. In contrast to the other studies, the use of laboratory faecal examination for parasites was a common practice in zoos to assess infection status and inform worming frequency; 81% of participating zoos used regular microscopic examination of faeces for GI parasites and 41% only wormed on evidence of infection. Faeces are widely used as a means to assess health and disease in zoo animals, when handling and physical examination are likely to be limited (Bishop *et al.*, 2015). However, this practice has limitations as an approach to assess GI parasite carriage and inform control. Intermittent shedding of eggs, pre-patent infection, low worm burdens and the inability to differentiate taenid and *Echinococcus* eggs morphologically can limit the value of faecal microscopy as a diagnostic tool, both in surveillance and to gauge treatment response. This could lead to an underestimation of infection rates in individual and groups of animals (Torgerson & Deplazes, 2009). Knowledge exchange involving researchers, zoo veterinarians and keepers and BIAZA is needed to explore ways to optimise worming and faecanalysis in zoo canid and hyaenid collections. Additional input should be sought from relevant working groups within the IUNC/SCC Canid Specialist Group, the chief body of scientific and practical expertise on the status and conservation of all canid species and the European Association of Zoos and Aquariums (EAZA) canid and hyaenid Taxon Advisory Group (TAG). The possibility of including optimal

evidence-based worming strategies into BIAZA membership and zoo inspection criteria should be considered. DEFRA should be consulted on the apparent lack of praziquantel worming requirements for canids imported to the UK under the Balai Directive (Council Directive 92/65/EEC) (Council of the European Union, 1992).

7.2.2.3. Faeces collection and disposal

All three canid studies identified routes of environmental faecal contamination, through voided faeces and methods of disposal of collected faeces.

A large proportion of participating farms (40.4%) reported not routinely collecting the faeces of their farm dogs despite over three quarters of farms (76.6%) reporting farm dog access to land shared with livestock. It is likely that these factors together contribute to contamination of pasture with *Echinococcus* eggs and an increased risk of livestock infection, particularly when considered with related factors of high risk ABP feeding, carcase scavenging and suboptimal worming. Significant associations were identified between dogs on a farm testing positive on *E. granulosus* G1 coproPCR and not collecting faeces at all ($p<0.05$) or not knowing if faeces were collected on farm ($p<0.05$). Despite prominent anti-fouling campaigns targeting the public to reduce dog fouling on farmland as a risk to livestock health (NFU, 2019; NSA, 2019b), there is evidence that this same advice is not being followed for working dogs on-farm.

Across the studies, 44.4% of zoos, 83% of farms and 50% of hunts that were collecting voided faeces were disposing of it by means e.g. muck heaps, slurry pits and farmer's fields, that could potentially contaminate agricultural and horticultural land if used as untreated compost or fertilizer, arguably over a wider area than would be contaminated by voided faeces alone. EU legislation classifies unprocessed manure as category 2 ABP and permits its use as compost providing it does not pose a risk to human or animal health (The European Parliament and The Council of European Union, 2011). However, the law is unclear on what constitutes risk and canid faeces do not conform to the legislation's definition of 'manure' as a product from farmed

animals. A clearer interpretation of the legislation relating to the use of untreated non-farmed animal faeces as compostable material is needed.

From an infection risk perspective, inadequate disposal of animal faeces and the potential spread of zoonotic disease is a public health issue that has not been assessed or controlled in most countries (Slifko, Smith and Rose, 2000). *E. granulosus* has been identified in samples of fruit and vegetables from European markets (Federer *et al.*, 2016a). There are few studies of the presence of infective *Echinococcus spp.* eggs in the environment (Slifko, Smith and Rose, 2000; Federer *et al.*, 2016b) and there are no specific studies addressing the effect of composting or sewage sludging on the viability of *Echinococcus* eggs (Eckert *et al.*, 2001). Fields fertilised with sewage contaminated with *T. saginata* eggs have been reported as an important source of infection to feedlot animals in the US (Cabaret *et al*., 2002). Based on experiments with ascarid eggs, it has been assumed that taeniid eggs would be killed by exposure of 30 mins or greater to temperatures exceeding 65˚C, generated within the sewage fermentation process (Eckert & Deplazes, 2004). It would be reasonable to assume that such conditions would not exist in all muck or compost heaps on farms. There are currently no systematic studies of the effect of sanitation technologies on the reduction of *Echinococcus* egg environmental contamination (Vuitton *et al.*, 2017). A study of *E. granulosus* egg survival in silage reported that oncospheres retained viability for up to 45 days (Pavlov *et al.*, 1969). However, ensiling methods have changed considerably and the current risk of feeding contaminated silage and its impact on parasite transmission is unknown (John, Davies, Williams, & Hodgkinson, 2019). Research is needed to address the knowledge gaps in understanding the risk posed by environmental contamination of *E. granulosus* eggs in the environment.

7.2.3. Evaluation of sensitivity and specificity of meat inspection for hydatid disease

A proof of concept exercise to evaluate meat inspection of hydatid cysts using molecular and histological methods as gold standard estimates high specificity and low sensitivity of inspection, potentially underestimating the true prevalence of hydatidosis in UK cattle.

Targeting livestock to prevent infection by optimising slaughter inspection, including liver/lung condemnations is an integral step of CE and echinococcosis control (Craig *et al.*, 2017). The abattoir study was able, for the first time, to explore the feasibility of using confirmatory PCR and histology testing of samples to evaluate and validate the MI process for the hydatidosis in livestock. As a pilot exercise and based on the total throughput for participating abattoirs in the study, a 2-stage approach estimated a diagnostic *Se* of 30.68% and *Sp* 99.48% for MI detection for hydatid lesions in cattle. Although there were limitations in the data available to calculate this estimate, the values are in broad agreement with a study using the reference gold standard to estimate a diagnostic *Se* of 24.9% and *Sp* of 98.9% for MI evaluation study of hydatid detection in cattle (Wilson *et al.*, 2019).

For MI detection of diseases that are a risk to public health or impact on animal welfare, it is important to limit the number of false negatives, i.e. have a high sensitivity of detection. With respect to limiting waste of meat products and economic loss at slaughter it is important to limit the number of false positives that could lead to unnecessary condemnations i.e. have a high specificity. These preliminary calculations suggest a low sensitivity of MI meaning that the true prevalence of hydatid disease in cattle (and possibly sheep also) is being underestimated, limiting effective disease condition reporting to farmers via the CCIR system. At a time when the Government is re-evaluating the way meat inspection results are reported to farmers to inform livestock management practice, presents valuable preliminary data how this process could be improved. Pending further revised calculations once individual abattoir throughput data are available to the study, these preliminary findings need to be discussed with the FSA, both as an indicator of hydatid lesion detection ability of the MI process and as proof-of-principle that laboratory testing is a useful tool to validate and improve the MI process and as a result, the CCIR reporting of disease.

7.3. Postal questionnaire and sampling survey design

All four thesis studies were based on a postal sampling and survey design. To the author's knowledge, this is the first time this method has been applied to

investigating echinococcosis in the UK. The postal survey allowed nationwide multi-site sampling of the four study populations for extended periods, which would not have been possible in-person by the researcher within the project time and resources. Sampling undertaken by personnel on-site avoided the additional logistical, health and safety and biosecurity implications of having the researcher attend sites in person, in particular zoos and abattoirs, where strict policies on on-site visitors will be in place (Bishop *et al.*, 2015; FSA, 2015.). Postal sampling hopefully avoided any other reservations participants may have had about researchers visiting sites in person.

A large-scale study of intestinal parasites in shelter dogs and cats in Canada adopted a similar postal sampling approach to collect 1700 faecal samples from 26 shelters around the country over a period of 18 months (Villeneuve *et al.*, 2015). The study reported cestode eggs in 1.6% of canine samples and *E. granulosus/E. canadensis* was detected by faecal flotation and multiplex coproPCR in samples from 4 dogs. The study reported similar limitations to the canine studies i.e. reliance on a single sample collection, intermittent parasite shedding, pre-patent infections at the time of sample collection and DNA extraction from low numbers of eggs, leading to reduced sensitivity of detection.

In the first instance, the author selected to collaborate with overarching associations rather than individual farms, hunts, zoos and abattoirs in order to increase the success of recruitment by obtaining support and endorsement from leader organisations, through which contacts and recruitment could be facilitated. Involving an interim association also allowed the introduction of an anonymisation step in the farm and abattoir studies, whereby the professional association could approach large numbers of members directly though their database, with only those agreeing to participate being revealed to the researcher. While this measure was taken to improve recruitment, it also involved more complex communications and organisation between stakeholders than contacting participants directly.

7.4. HyData study recruitment

The hunting hound and farm dog studies had aimed to calculate apparent prevalence and freedom from disease at a regional level. A lower than expected study sample size meant that these inferential analyses could not be performed. However, the studies yielded valuable descriptive data on the distribution of *E. granulosus* and *E. equinus* infection in the UK and on risk factors for infection.

All the studies in the thesis represent forms of purposive sampling, in that they represent study subjects with known exposure to risk factors i.e. the canine studies or a specific disease status i.e. the abattoir study. In the farm dog study, a randomisation step was introduced to try and limit systematic error and attempt probability sampling within the subset of the source population. Furthermore, the studies recruited members of associations upholding best practice within their fields, so participants willing to take part may represent those already demonstrating higher standards of animal health and biosecurity. As such, husbandry and management practices in the wider farm, zoo and hunt communities, and their associated risk of *Echinococcus* spp. transmission, may have been underreported in the studies.

The thesis has benefited from collaboration with four national-level organisations; the FSA, the NSA, BIAZA and the Hunting Office. The infrastructure, communication networks and support of these collaborators have allowed the project to undertake far-reaching research at the national level within the time and resources available. However, this also led to logistical challenges, in particular relating to the abattoir work, which required a complex process of abattoir recruitment and information exchange via the FSA. Delays and problems relating to the sensitive nature of baseline throughput data for the abattoirs, meant that these data could not be obtained and incorporated into the validation exercise during the timeframe of the project. The FSA did provide an overall throughput figure for the 15 participating abattoirs, from which estimate analyses on the sensitivity and specificity of MI using the existing dataset could be made. The value of the pilot exercise, developed after discussions with the FSA identified a need and opportunity to explore the validation of the MI process, was reduced. However, the exercise has yielded useful preliminary

data from which further work on a test validation paradigm can be explored. The abattoir study must also take into consideration limitations relating to baseline data; firstly, only a low proportion of the expected hydatid lesions that were identified at participating abattoirs (12.3%) were received by the study. The instruction to participating sites was to submit all hydatid lesions identified on inspection. Poor compliance with the study instructions mean that the sample collection does not reflect the type, number and species distribution of lesions seen at these abattoirs, nor the larger picture of hydatidosis in UK livestock as a whole.

7.5. Laboratory methods

The detection of Echinococcus parasites in the faeces of definitive hosts can be challenging (Boufana *et al.*, 2015) especially in study areas of low prevalence, when the sample population is regularly or intermittently wormed and samples are collected from voided faeces in the environment (Lett, 2013). These challenges applied to all the canine studies in the thesis that used canid faeces as the starting material for analysis.

Neither coproELISA or coproPCR represent gold standard methods for the diagnosis of *Echinococcus* infection in dogs, and both have a number of limitations affecting sensitivity and specificity. In this study, Cohen's Kappa statistic to assess the agreement of the tests beyond chance showed only slight agreement between the tests. More recently, test accuracies in the absence of a gold standard for *Echinococcus* coprodiagnosis in dogs have been estimated using latent class analysis (Hartnack, Budke, Craig, *et al.*, 2013; Craig *et al.*, 2015; Otero-Abad *et al.*, 2017). Bayesian latent class models allows true prevalence to be estimated using multiple tests in parallel, taking into account influencing factors that can affect sensitivity and specificity of the tests used. Such methods have been used successfully to explore the epidemiology of canine echinococcosis in highly endemic settings (Hartnack *et al.*, 2013; Otero-Abad *et al.*, 2017; Ziadinov *et al.*, 2008). Due to the very low number of positive samples returned in these studies, these methods were not suitable for application here.

In this study, interpretation of the coproELISA relies on a single cut-off value to determine negative samples (below the cut-off) from positive samples (above the cut-off). The cut off value has been determined using the Gaussian distribution method (J C Allan et al., 1992; Christofi et al., 2002) primarily designed to identify negative samples, which are presumed to follow a Gaussian distribution, resulting in a sensitivity of 99.9%. A limitation of this method is the lack of consideration of the distribution of positive samples in determination of the cut-off value. The cut off value is determined solely on a panel of negative samples and does not include the distribution of a panel of positive samples. It is assumed that there would be no cross reactions with other taeniid species that could affect the Gaussian distribution of the negative control panel and hence the selected cut off OD. The discriminatory ability of the test relies on establishing an optimal signal:noise ratio, as has been performed for the coproELISA test used in this study. Should a panel of known positive samples from a low-endemicity setting have been available, an improved method of calculating the cut-off value using Receiver Operator Characteristic (ROC) curves could have been used. This method considers the distribution of both negative and positive samples when calculating a cut-off value (Gardner & Greiner, 2006; van Kesteren, 2015) and can allow varying of the cut-off point, to tailor the sensitivity and specificity of the assay according to the requirements of the test (Fan, Uphadyhe, & Worster, 2006; Gardner & Greiner, 2006; Otero-Abad & Torgerson, 2013; van Kesteren, 2015).

CoproELISA sensitivity can be affected by the distribution of parasites within the community tested, with low worm burden animals generally having a lower OD value (Buishi *et al.*, 2005; Jenkins *et al.*, 2006). As overall low endemicity is expected in the UK, it is possible that the sensitivity of the coproELISA tests has been affected by all animals potentially having a low worm burden (Craig *et al.*, 2015; Deplazes *et al.*, 1992). A threshold of detection of approximately 50-100 worms for the coproELISA test has been suggested in the literature, though not validated using the Gaussian distribution method (Craig *et al.*, 2015; Deplazes *et al.*, 2011).

Samples testing positive on coproELISA but negative on coproPCR may be at the prepatent stage of infection (Craig *et al.*, 2015). It is possible that intermittent

anthelmintic treatment could reduce the chance of worms being at a patent stage of infection, thus shedding eggs, at the time of sampling. Praziquantel is a highly effective cestocidal drug, but has no residual effect and will offer no protection against reinfection (Lembo *et al.*, 2013). Furthermore, an expected low worm burden, and resultant low egg number in faeces, could mean that the measured sample of faeces used for analysis did not reach the 1 egg per gram of faeces detection threshold for the coproPCR protocols used here (Boufana *et al.*, 2013; Lett *et al.*, 2018). Conversely, samples testing positive on coproPCR but negative on coproELISA may be below the detection threshold of the coproELISA assay (false negatives) or may be contaminated with *Echinococcus* spp. eggs ingested through coprophagia (Hartnack, Budke, Craig, *et al.*, 2013).

Molecular methods such as PCR are of particular importance in species identification given that eggs from *Echinococcus spp*. cannot be differentiated morphologically from other taeniid eggs (Craig *et al.*, 2015). CoproPCR presents a valuable method of standalone or adjunctive identification and allows downstream sequencing and phylogenetic study. However, faeces contain a variety of inhibitory substances that can interfere with test efficacy (Ito, 2013). Substances in faeces such as tannic acid, humic acid and calcium can prevent successful amplification of target DNA, adding to the challenges of successful coproPCR (Schrader *et al.*, 2012). Optimisation steps, including addition of formamide and BSA to enhance DNA amplification and sample dilution to reduce inhibitor concentration were used in all coproDNA assays. PCR inhibition may have affected molecular detection in the canine studies, where low prevalence and the overdispersed nature of infection may result in low levels of parasite-derived DNA in sample material.

Extracted DNA from canine faecal samples will contain genetic material derived from the host, the host's recent animal and/or plant-based ingesta, commensal and invasive gut bacteria, gut parasites and other environmental contaminants. Molecular faecanalysis protocols may improve sensitivity by concentrating parasite egg or adult material upstream of DNA isolation and PCR processes by sieving (Mathis, Deplazes and Eckert, 1996) or zinc chloride flotation (Cabrera *et al.*, 2002), which also isolates parasite material from faecal PCR inhibitory material. In the UK,

the low endemicity of canine infection and expected low numbers of eggs present in the faeces of infected canines precludes egg isolation or concentration steps, where scant target material may be lost in the process. Furthermore, the coproPCR method used here by Abbasi *et al.* (2003) has demonstrated detection of infection in the pre-patent period (21-25 days post-infection) of experimentally infected dogs (Lahmar *et al.*, 2007). The ability to employ coproPCR methods validated for faecal samples without prior isolation of taeniid eggs is an advantage if a large number of samples require testing (Craig *et al.*, 2015), as in the HyData study. For these reasons, to increase the chances of correct identification of PCR positive samples in a challenging low-endemic setting, it was decided to perform DNA extraction on whole faeces without techniques to concentrate or isolate parasite eggs.

The study numbers obtained in the thesis permitted both coproPCR and coproELISA methods to be used for all the study samples submitted. The ability to use both methods can provide supporting data and increase the confidence in the specificity and sensitivity of an epidemiological data set (Craig *et al.*, 2017) particularly in this low endemicity setting (Christofi *et al.*, 2002). From a public health perspective, species-specific detection and differentiation of the two endemic UK species has been important, as only *E. granulosus* is considered zoonotic. For larger surveillance studies, the labour and cost of PCR testing may preclude its use as a first line screening tool (Craig *et al.*, 2015, 2017). In such cases, screening a proportion of coproantigen positive and negative samples can still be useful a useful adjunct, although there is evidence that the tests do not always agree when used in sequence (van Kesteren, 2015).

The HyData studies used a combination of genus- and species- specific molecular diagnostic methods. There was mixed success in the performance of the coproPCR tests. Unfortunately, the genus-specific protocols by Trachsel *et al.* (2003) and Abbasi *et al.* (2003) (Abbasi *et al.*, 2003; Trachsel, Deplazes and Mathis, 2017) did not perform well in the studies despite troubleshooting. However, species-specific protocols by Boufana *et al.* (2013) and Lett *et al.* (2013) did produce consistent results and allowed species identity based on the presence of a specific size band on gel electrophoresis. PCR amplicon sequencing did not produce sequences of sufficient

quality for further confirmation of identity, a known limitation of the procedure (Lett, 2013a; van Kesteren, 2015). Further work is needed to optimise DNA extraction and PCR, especially in low prevalence setting to obtain a product suitable for sequencing.

7.6. Conclusions

In conclusion, the thesis has yielded strong evidence, based on molecular testing in definitive and intermediate host populations, of the spread of the zoonotic parasite *E. granulosus* to previously unreported areas of the UK. As an exercise in baseline data gathering, the approach of the thesis and its findings would inform the planning phase of a UK hydatid control programme (Craig *et al.*, 2017).

The studies have yielded a number of important descriptive findings that challenge our current understanding of the distribution of *E. granulosus* and *E. equinus* in the UK and point to further areas of research and intervention where control measures are lacking and opportunities for transmission occur. The significance of evidence that a zoonotic pathogen is present in parts of the UK thought to be non-endemic cannot be underestimated.

Together, the four HyData studies have investigated the epidemiology of echinococcosis in the UK and has found evidence of widespread infection of definitive and intermediate host populations with *E. granulosus*, challenging the idea that this zoonotic parasite is confined to known areas of endemicity. These findings call for further urgent research to establish the significance of these detections and whether they reflect other regions of high prevalence in the UK, including a possible first case in Northern Ireland. The next steps involve wider studies of surveillance to fill gaps in our knowledge on prevalence. This will allow more targeted and evidence-based interventions. The steps needed to reduce transmission of *E. granulosus* are simple, known and actionable. They focus on adequate risk-based approach to worming of domestic dogs, avoidance of feeding and scavenging opportunities of high-risk infective raw food materials and adequate collection and disposal of dog faeces. The success of such measures, when applied effectively has been evidenced in control programmes around the world, including the UK. However, it is clear from the findings of the thesis that there are still practices that perpetuate parasite transmission and areas of much needed intervention, if the growing evidence of a re-emergent and emergent picture of *E. granulosus* spread in the UK is to be addressed.

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APPENDICES

I. Appendix I: Chapter 3 material

Appendix I-a. Summary of UK hunts by hunt type and status as registered in Baily's Hunting directory in May 2019.

Hunt	England	Wales	Scotland	NI	Total
Foxhound					
Registered	153	34	11	6	204
Active	150	34	10	6	200
Merged	7	0	0	0	7
Disbanded	3	0	1	0	4
Beagles					
Registered	63	6	0	3	72
Active	58	5	0	3	66
Merged	1	0	0	0	1
Disbanded	5	1	0	0	6
Bloodhounds					
Registered	22	3	0	2	27
Active	18	2	0	2	22
Merged	0	0	0	0	0
Disbanded	4	1	0	0	5
Minkhounds					
Registered	19	4	1	0	24
Active	17	4	1	0	22
Merged	0	0	0	0	0
Disbanded	2	0	0	0	2
Draghounds					
Registered	11	3	0	0	14
Active	6	3	0	0	9
Merged	0	0	0	0	0
Disbanded	5	0	0	0	5
Harriers					
Registered	20	0	0	7	27
Active	18	0	0	7	25
Merged	0	0	0	0	0
Disbanded	2	0	0	0	2
Bassets					
Registered	8	3	1	0	12
Active	8	3	1	0	12
Merged	0	0	0	0	0
Disbanded	0	0	0	0	0
Staghounds					
Registered	3	0	0	1	4
Active	3	0	0	1	4
Merged	0	0	0	0	0
Disbanded	0	0	0	0	0
Rabbit dogs					
Registered	1	0	0	0	1
Active	1	0	0	0	1
Merged	0	0	0	0	0
Disbanded	0	0	0	0	0
Total registered	300	53	13	19	385
Total active	279	51	12	19	361
Total merged	8	0	0	0	8
Total disbanded	21	2	1	0	24

Appendix I-b. letter of introduction, outlining the design, aims and objectives of the study was sent to the Hunting Office, the Secretary of each of the hunt associations in England, Wales and Scotland and the chairman of the Hunting Association of Ireland

Department of Epidemiology & Population Health,
Institute of Infection & Global Health,
University of Liverpool.

Leahurst Campus
Chester High Road
Neston
Cheshire CH64 7TE

Telephone: +44 (0) 151 794 6040
Email: collinsm@liv.ac.uk

March 2016

Dear (Master of Hounds),

***Hy*Data UK 2016: A Study of *Echinococcus granulosus* tapeworm in the UK**

I am writing to inform you of a national study to investigate the distribution of the dog tapeworm, *Echinococcus granulosus,* taking place in 2016. My name is Marisol Collins and I am a veterinary surgeon undertaking this research at The University of Liverpool. This parasite is a cause of considerable economic loss to the UK livestock industry and poses an infection risk to many species, including, dogs, horses and humans. Little is known about the current national distribution of the parasite, and recent pilot research suggests that it is more widespread than previously understood. The study is led by a research team at The University of Liverpool in collaboration with The University of Salford, and is funded by the Biotechnology and Biological Sciences Research Council (BBSRC), a Government-funded research agency, and Bayer plc. The study, the most comprehensive of its type, has the support of Professor The Lord Trees, cross-bench peer in The House of Lords and a distinguished expert in parasitology.

It is very important to the research team that the respective Masters of Hounds are fully aware and support the study ahead of its commencement this Spring. As leaders within the sport of hunting with hounds, we would also greatly appreciate any input or comments regarding the study that you may have. This research forms part of a larger national study investigating the distribution of the tapeworm in hunting hounds, farm dogs, zoos and livestock in the UK, which will complete in 2019. The study aims to send a questionnaire to all hunt kennels, addressed to the Kennel Master, to gather information about husbandry, feeding practices and de-

worming protocols of the hounds. A kit will be provided to collect a number of faeces samples from the hounds (to be returned by post or collected on a pre-arranged visit with a project veterinary investigator) for analysis of *Echinococcus granulosus* DNA. Involvement in the study is completely voluntary and participants can withdraw from the study at any time. All data gathered for the study will be **fully anonymized**, with no hunt, individuals or hounds identifiable from the analysed data. All data gathered will be safely stored at a secure University database. The study aims to build a national picture of parasite distribution, which will help to advise optimal approaches to parasite control and public health. On completion of the research, participating hunts will be informed of the overall findings and recommendations, which could help to optimise your parasite control programme, public safety and overall health of your hounds.

We would be grateful if you would accept a telephone call from our lead veterinary investigator, Ms Marisol Collins, who will be happy to discuss any aspects of the study ahead of questionnaire deployment. We will aim this to take place within 2 weeks of receipt of this letter. This is an opportunity for you to raise any comments or queries regarding the study, its purpose and proposed outcomes. Alternatively, if you do not wish to endorse the study, and do not wish to be contacted, please reply by email or post to the given address inform us of your wishes.

Your support and participation is greatly appreciated. Thank you for taking the time to read our material, and we look forward to hearing from you soon.

Yours sincerely,

Marisol Collins BSc BVSc MSc MRes MRCVS

Appendix I-c. Participant information sheet

_Hy_Data Participant Information Sheet

***Echinococcus* tapeworm infection in livestock and high-risk dog populations**

You are invited to take part in a survey about the husbandry and health care of hunting hounds. This forms part of a larger 3-year research project into tapeworm infections in dogs and livestock in the UK. It is important that you understand fully the purpose, process and outcomes of this research before deciding if you wish to take part. **Please read this information sheet carefully and feel free to raise any questions, comments or request clarification about any part of the study.**

What is the purpose of this study?

This questionnaire is part of a research study on ***Echinococcus***, a tapeworm parasite of dogs, which can also infect livestock, horses and people. This parasite is present in the UK, but information about where in the country it is found is currently lacking.

The tapeworm does not pose a health risk to dogs, but infection spread by them to livestock, such as sheep and cattle can lead to significant production losses for livestock farmers. In the rare event that a person is infected, this can result in debilitating disease that is difficult to treat.

Through our study, we hope to find out more about *Echinococcus* in dog populations and livestock. We are including hunting hounds because this is a well-defined dog population. Our study will also include sampling *Echinococcus* in farm dogs, sheep, cattle and horses in the UK. With this information, we aim to improve and inform on the welfare, safety and health of people, domestic animals and livestock in our country.

Who is conducting the study and who is it funded by?

The study is led by researchers at the Institute of Infection and Global Health at The University of Liverpool and The University of Salford. The study is funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and Bayer plc. The study is conducted with full ethical approval from The University of Liverpool and with the knowledge of the respective hound associations representing the hunts taking part.

Why have I been chosen to take part?

All hunting packs currently registered in *Bailys Hunting Directory* are invited to take part in the study. You have been chosen to take part as you are principally in charge of the care and welfare of the hounds in your Hunt.

What does taking part involve?

You are being invited to help in two ways; firstly, to complete a questionnaire, which should take no more than 10 minutes. Secondly, you are asked to submit a sample of faeces from the hounds, via a sampling kit we provide free-of-charge, or by prior arrangement for collection by the lead veterinary investigator in the study. No direct contact with the dogs is involved in the study.

Do I have to take part?

Taking part is completely voluntary and you are free to withdraw from the study at any time.

1

Will taking part in the study be confidential?

Yes. Any information you give will be held in strict confidence in a secure, password-protected database at the University of Liverpool in compliance with the Data Protection Act (1998). All information will be **fully anonymised** and will be destroyed within 5 years of study completion. Questionnaires will be identified by a unique code, which will be used to link samples to the right questionnaire and ensure that further invitations are not sent to existing participants. Your personal details, those of the hunt and hounds will not be identified through published research. **By completing this questionnaire and providing samples, you are giving informed consent for your data to be included in the study.**

What will happen to the information and samples I provide?

The questionnaire data will be safely stored and later analysed to investigate the national distribution of *Echinococcus*, and possible risk factors for infection in a rural setting. Faecal samples from the hounds will be tested in the laboratory to see if they contain the *Echinococcus* parasite. At the end of the study we cannot provide individual participant results, but a copy of the study report will be given to each participating Hunt.

What are the benefits and risks of taking part in the study?

Collection of samples is not expected to cause any undue health risks above those encountered during your normal daily care of the hounds. We do recommend that you use the disposable gloves provided during sample collection and follow the instructions as show. Taking part will help us gather vital information that may help protect your hounds and horses, the livestock and people that share our Countryside from a potentially harmful parasite.

What will be the outcome of the study?

We aim to publish the results of the study in appropriate scientific journals, and present the findings at scientific meetings. No participants will be identifiable from any published or presented results.

What if I no longer wish to take part?

If you change your mind and no longer wish to take part, even after you have answered the questionnaire and submitted samples, we will delete your answers and destroy the samples so that they are not included in the study. Simply contact us to request this.

What should I do if I have a problem?

If you are unhappy with any aspect of the study or encounter a problem, please inform the lead researcher, Marisol Collins (details below) or the Principal Investigator, Dr. Phil Jones on 0151 795 6056 and we will endeavour to help. If you have a complaint that you do not wish to share with the project team, please contact the Research Governance Officer of The University of Liverpool at ethics@liv.ac.uk with the name and description of the study, so it can be identified.

Who can I contact if I have further questions?

If you have any questions or comments relating to the study, please contact the veterinary researcher:

Ms. Marisol Collins BVSc MSc MRes MRCVS
Institute of Infection and Global Health
University of Liverpool
Leahurst Campus
CH64 7TE
Tel: 0151 795 6040
hydata@liv.ac.uk

Thank you for your valuable contribution

2

Appendix I-d. Study questionnaire

UNIVERSITY OF LIVERPOOL

*Hy*Data UK Survey 2016

Thank you for agreeing to take part in this study. Your participation is greatly valued and will help us to better understand the distribution of the *Echinococcus* tapeworm in dogs and livestock in the UK.

As part of our research, we are inviting all registered Hunts in England, Scotland, Wales and Northern Ireland to complete a questionnaire about the husbandry and healthcare of their hounds. In addition, we are asking for permission to allow a sample of faeces from the hounds to be collected.

Before completing the survey, please read the accompanying Participant Information Sheet carefully, complete the Participant Consent Form attached to this questionnaire and read the instructions below.

How to complete the survey

The survey is divided into two sections and should take approximately 10 minutes to complete:

Section 1: The Hunt Pack Here, questions relate to the Hunt, the number of hounds, their breeds, sex and whether any hounds from another Hunt have recently been resident at the kennels.

Section 2: Husbandry at The Kennels This section includes questions about the diet of the hounds, their feeding regime, exercise facilities and deworming procedures.

Please answer questions in BLOCK CAPITALS in the text box provided **OR** by circling the appropriate answer if asked YES / NO / Don't know **OR** by placing a tick in the box ☑ where appropriate. If you change your answer or make a mistake, please fill in the first box completely ■ and put a clear tick in the correct box.

Taking part in this study is entirely voluntary and you are able to withdraw at any time. All information collected in the survey is strictly confidential. All data will be anonymized and no individual persons or hounds will be identifiable from any published results. Questionnaires will be identified by a unique code, which will match questionnaires and samples and will be used to ensure that further invitations are not sent to existing participants.

Please place the completed questionnaire in the pre-paid addressed envelope provided for return to the research team. Thank you.

Date DD / MM / 2016

Section 1: The Hunt Pack

For official use only

1. Name of the Hunt

2. Have hounds from another Hunt been temporarily housed at your kennels in the last 12 months? *(please circle the appropriate answer)* YES / NO/ Don't know

3. If so, please give the name of this Hunt and the number of hounds:

*The remaining questions in the questionnaire only concern hounds and terriers belonging to **your** Hunt Pack.*

4. What breed(s) are the hounds?

5. What breed(s) are the terriers?

6. Please complete the number of hounds and terriers in the table below (entering number of individual dogs NOT couples):

	Total	Males (over 6 months old)	Females (over 6 months old)	Pups (under 6 months old)
Hounds				
Terriers				

Section 2: Husbandry at The Kennels

1. How many meals are fed to the hounds over 24 hours?

2. Are the kennels licensed as a Collection Centre for Animal By-Products?
YES / NO / Don't know

3. Within the last 12 months, please indicate which of the following diets have been fed to the hounds *(tick all that apply):*

	Sheep	Lamb	Beef	Calf	Pig	Horse	Donkey	Goat	Poultry
Raw flesh from fallen stock (whole or part carcass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw liver or lungs (lights) from fallen stock	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw flesh from abattoir/butcher	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw liver or lungs (lights) from abattoir/butcher	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cooked flesh from butcher or boiled on-site	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cooked liver or lungs (lights) from butcher or boiled on site	☐	☐	☐	☐	☐	☐	☐	☐	☐

Commercial dog food (dry/canned) ☐ Catering waste ☐

Fish ☐ Other (please specify) ☐

4. If used, please indicate how you store RAW meat intended for the hounds in the flesh house *(tick all that apply)*:

Freezer ☐

Refrigerator ☐ Other *(please specify)* ☐

Room temperature ☐

5. How does the Hunt dispose of Specified Risk Material (SRM) and raw meat waste?

Incinerator on-site ☐ Other *(please specify)* ☐

Rendering plant ☐

Incinerator off-site ☐

For official use only

For official use only

6. Please describe the environment given for the hounds to exercise (not including hunting) *[please tick all that apply]*:

Fenced grass yard for hounds only	☐	Fenced field/area shared with livestock	☐
Fenced concrete yard for hounds only	☐	Other *(please specify)*	
Open land with public access	☐		

7. Are the hounds dewormed regularly as part of a 'Hound Health Programme'?

YES / NO / Don't know

8. Please complete the table below about **any** deworming undertaken at the kennels:

	Hounds	Whelping bitches	Pups	Terriers
Name of dewormer used				
Dose given (e.g. mg/kg, ml/kg or tablets/dog)				
Frequency of deworming (e.g. times per week/month/year)				
Date of last deworming (DD/MM/YY)				
Person responsible for deworming (e.g. kennelman, huntsman, vet)				

9. How are hound faeces disposed of at the kennels?

Please describe collection routine, method and disposal site

10. Is the 'Code of Practice for the Welfare of Hounds in Hunt Kennels' document available to the Hunt? YES / NO / Don't know

11. Would you be willing to kindly participate in a second part of this study by collecting faeces samples from the hounds using the kit provided? The samples are returned **FREE OF CHARGE** by post for **anonymized** testing for *Echinococcus* tapeworm.

YES ☐ NO ☐

12. Would you be willing to kindly allow the lead investigator to visit the kennels to collect a further set of faeces samples from the hounds at a pre-arranged date and time for **anonymized** testing for *Echinococcus* tapeworm?

YES ☐ NO ☐

End of survey. Thank you for your valuable contribution. Your help is greatly appreciated.

Appendix I-e. Summary table of associations between coprodiagnostic test outcomes and questionnaire variables

		CoproELISA		Any coprotest positive	
Diet type		**Number of hunts**	**P-value**	**Number of hunts**	**P-value**
Faeces disposal					
	Rendering plant	0	1	0	1.000
	Muck heap	1	1	2	0.620
	with SRM	0	1	0	1.000
	Offal skip	0	1	0	1.000
	Burning on site	1	0.181	1	0.238
	Farmer	1	0.181	1	0.238
	Waste disposal company	0	1	0	1.000
	Slurry pit	0	1	0	1.000
	Refuse bin	0	1	0	1.000
Region	All	3	0.732	4	0.834
	Scotland	1	0.34	1	0.431
	Wales	0	1	0	1.000
	England	2	1	3	1.000
	Northern Ireland	0	1	0	1.000
Worming	Contain praziquantel	1	0.568	2	1.000
	According to manufacturer	0	0.555	0	0.550

Appendix I-f. frequency of feeding, type and source of food for the hounds

		CoproELISA		Any coprotest positive	
Diet type		**Number of hunts**	**P-value**	**Number of hunts**	**P-value**
Fallen stock (Meat)					
	Cattle/Sheep	3	0.541	4	0.303
	Other mammals	3	0.253	4	0.128
	Poultry	0	1.000	0	1.000
	Equid	3	0.253	4	0.128
Fallen Stock (Viscera)					
	Cattle/Sheep	2	0.568	2	1.000
	Other mammals	2	0.224	2	0.572
	Poultry	0	1.000	0	1.000
	Equid	2	0.224	2	0.572
Abattoir/butcher (Meat)					
	Cattle/Sheep	1	0.536	2	0.201
	Other mammals	1	0.478	1	1.000
	Poultry	1	1.000	0	1.000
	Equid	1	0.094	1	0.125
Abattoir/butcher (Viscera)					
	Cattle/Sheep	1	0.263	1	0.340
	Other mammals	1	0.094	1	0.125
	Poultry	NA	NA	NA	NA
	Equid	NA	NA	NA	NA
Cooked (Meat)					
	Cattle/Sheep	0	1.000	0	1.000
	Other	NA	NA	0	1.000
Cooked (Viscera)					
	Cattle/Sheep	0	1.000	0	1.000
	Other	NA	NA	NA	NA
Other	Commercial	1	0.224	2	0.572
	Catering waste	1	0.094	1	0.125
	Fish	0	1.000	0	1.000
	Other	0	1.000	0	1.000
	VH risk diet	3	0.541	4	0.303
	Any risk diet	3	1.000	4	0.552
	Low risk only	0	1.000	0	1.000
	E. equinus risk	3	0.253	4	0.128
Raw food storage					
	Freezer	0	1	0	1.000
	Refrigerator	0	1	0	0.545
	Room temperature	3	1	4	0.561
	Dried by fan	0	1	1	0.495
	Coolbox	0	1	0	1.000
Fallen stock disposal					
	Incinerator on site	NA	NA	NA	NA
	Incinerator off site	1	0.356	1	0.453
	Return to supplier	0	1	0	1.000
	Rendering plant	2	0.453	3	1
	NFSCo collection centre	3	0.238	3	0.613
Sites of access					
	Fenced grass yard	1	0.224	2	0.572
	Fenced concrete yard	2	1	2	1.000
	Fenced field/area with live	1	1	3	1.000
	Open land with public acce	1	0.224	2	0.572
	Roads	0	0.555	0	0.550
	Potential livestock/public ri	2	0.094	3	0.125

II. Appendix II: Chapter 4 material

Appendix II-a. Letter of support from the BIAZA Research Committee for the HyData zoo project

BIAZA Research Committee
Letter of Support for Research Project

The BIAZA Research Committee promotes good quality basic and applied research by and within BIAZA's member collections.

Following critical consideration of the research proposal and subsequent satisfactory responses by the researcher, the committee has agreed to give a letter of support for this study by Elizabeth Attree of the University of Liverpool.

In the opinion of the BIAZA Research Committee the outcomes of the project are likely to be relevant and useful to zoos and aquariums

In the interest of scientific training [and the furthering of science], the BIAZA Research Committee encourages BIAZA members to take part in this research project.

Please be advised that we would require an update or your completed project report within 1 year from today.

Yours faithfully,

Appendix II-b. HyData zoo study participant information sheet issued with the questionnaire and sampling kits.

UNIVERSITY OF LIVERPOOL

Participant Information Sheet

A survey of gastrointestinal (GI) parasites of canines and hyenas in UK zoos and wildlife parks, with a focus on** Taenia **and** Echinococcus **tapeworm species.

You are invited to take part in a survey about gastrointestinal parasites in UK canine and hyena zoo collections, with a focus on tapeworms of public health importance. This forms part of a Masters research project and a wider PhD study of tapeworm infections UK animal populations. It is important that you understand fully the purpose, process and outcomes of this research before deciding if you wish to take part. **Please read this information sheet carefully and feel free to raise any questions, comments or request clarification about any part of the study.**

What is the purpose of this study?

Canine and hyena species can carry GI parasites of public and animal health importance, including zoonotic *Taenia* and *Echinococcus* tapeworm species. The specialist husbandry of such carnivores in zoos includes the feeding of raw meat and whole livestock carcasses. To provide such diets, zoos can register as collection centres of animal by-products (ABP) from the livestock slaughter industry. A derogation within the law permits zoos to source categories of ABP for animal feed that may contain pathological lesions indicating disease communicable to man or animal. This poses a possible transmission risk if raw meat and offal from sheep and cattle infected with parasites prior to slaughter are then fed to competent captive hosts.

To our knowledge, the risk of zoonotic transmission to zoo personnel via infective eggs passed in carnivore faeces in the UK has not been explored. Of particular interest is *Echinococcus granulosus*, a tapeworm parasite of dogs, which cannot be reliably identified by routine faecal microscopy and can infect many species including humans; it is present in the UK but information about where in the country it is found is currently lacking.

Through our study, we hope to find out more about overall GI parasite burden in an important group of carnivore mammals, and investigate this with reference to diets fed and worming treatments used at different premises. Our overarching study includes zoo species because this is a well-defined animal population at risk, alongside our work on farm dogs, hunting hounds, sheep and cattle. With this information, we aim to improve and inform on the welfare, safety and health of people, zoo and domestic animals and livestock in the UK.

Who is conducting the study and who is it funded by?

The study is led by veterinary researchers at the Institute of Infection and Global Health at The University of Liverpool and The University of Salford. It is funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and Bayer plc. The study is conducted with full ethical approval from The University of Liverpool and with the knowledge and support of the British and Irish Association of Zoos and Aquariums (BIAZA).

Why have we been chosen to take part?

All zoo collections in England, Wales and Scotland have been considered for the study, whether they hold BIAZA membership or not. You have been chosen to take part as your collection stocks species of interest to the study.

What does taking part involve?

You are being invited to help in two ways; firstly, to complete a short questionnaire, which should take no more than 10 minutes. Secondly, you are asked to submit samples of voided faeces from species of interest, via a sampling kit we provide free-of-charge and returned via pre-paid post. No direct contact with animals is involved in the study.

Do I have to take part?

Taking part is completely voluntary and you are free to withdraw from the study at any time.

1

Will taking part in the study be confidential?

Yes. Any information you give will be held in strict confidence in a secure, password-protected database at the University of Liverpool in compliance with the Data Protection Act (1998). All information will be **fully anonymised** and will be destroyed within 5 years of study completion. Your personal details, and those of any zoo staff assisting with the sampling, will not be identified through published research. **By completing this questionnaire and providing samples, you are giving informed consent for your data to be included in the study.**

What will happen to the information and samples I provide?

The questionnaire data will be safely stored and later analysed to explore the husbandry and GI parasite treatment of the zoo species of interest. Faecal samples will be examined by microscopy to identify the types of parasites found and further analysed using molecular methods to see if they contain *Taenia* and/or *Echinococcus* DNA. At the end of the study we will report the results of the laboratory investigation to you and would be interested to discuss these in light of the parasite control measures you have in place.

What are the benefits and risks of taking part in the study?

Collection of samples is not expected to cause any undue health risks above those encountered during your normal daily care of the animals. We recommend that you use the disposable gloves provided with the sampling kit during sample collection, follow the instructions as shown and seek medical advice should an injury or spillage occur during, or you feel unwell after sampling. We hope that data generated by the study will provide valuable information to zoos about preventative health measures and parasite control, and putative public health risks in the event of zoonotic parasite carriage. Taking part will also help us gather vital information about the UK distribution of parasites that are important to animal and public health.

What will be the outcome of the study?

We aim to publish the results of the study in appropriate scientific journals and present the findings at scientific meetings. No zoos will be named in any published or presented results, however, if you house a species uncommon to UK collections, your zoo may be indirectly identifiable from results. Please discuss this with the research team if you have concerns.

What if I no longer wish to take part?

If you change your mind and no longer wish to take part, even after you have answered the questionnaire and submitted samples, we will delete your answers and destroy the samples so that they are not included in the study. Simply contact us to request this.

What should I do if I have a problem?

If you are unhappy with any aspect of the study or encounter a problem, please inform the lead researcher, Marisol Collins (details below) or the Principal Investigator, Dr. John McGarry on 0151 794 1518 and we will endeavour to help. If you have a complaint that you do not wish to share with the project team, please contact the Research Governance Officer of The University of Liverpool at ethics@liv.ac.uk with the name and description of the study, so it can be identified.

Who can I contact if I have further questions?

If you have any questions or comments relating to the study, please contact the veterinary researchers:

Ms. Marisol Collins BVSc MSc MRes MRCVS
Institute of Infection and Global Health
University of Liverpool
Leahurst Campus
CH64 7TE Tel: 0151 795 6040
collinsm@liverpool.ac.uk

Ms. Elizabeth Attree BSc
Institute of Infection and Global Health
University of Liverpool
Leahurst Campus
CH64 7TE Tel: 0151 795 6040
hleattre@liverpool.ac.uk

Thank you for your valuable contribution

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Appendix II-c. HyData zoo study questionnaire for a survey of Echinococcosis in captive canids and hyaenids in UK zoos

UNIVERSITY OF LIVERPOOL

*Hy*Data Zoo UK Survey 2018

Thank you for agreeing to take part in this study. Your participation is greatly valued and will help us to better understand the distribution of the *Taenia* and *Echinococcus* tapeworms in captive canids and *hyaenidae* in the UK.

As part of our research, we are inviting Zoos and other animal collections in England, Scotland and Wales to complete a short questionnaire about the husbandry and healthcare of their canids and *hyanidae*. In addition, we are asking for a sample of faeces from the animals to be collected by keepers or carers of the species. If your collection has more than one species of interest we will ask for a separate questionnaire and sample to be completed per species.

Before completing the survey, please read the accompanying Participant Information Sheet carefully, complete the Participant Consent Form attached to this questionnaire and read the instructions below.

How to complete the survey

The survey is divided into two sections and should take approximately 10 minutes to complete:

Section 1: The animals: In this section questions relate to the species, number of animals, their sex and whether any animals from another zoo have recently been transferred to the zoo.

Section 2: Husbandry at the zoo This section includes questions about the diet of the animals, their feeding regime, enclosure and deworming procedures.

Please answer questions in BLOCK CAPITALS in the text box provided **OR** by circling the appropriate answer if asked YES / NO / Don't know **OR** by placing a tick in the box ☑ where appropriate. If you change your answer or make a mistake, please fill in the first box completely ■ and put a clear tick in the correct box.

Taking part in this study is entirely voluntary and you are able to withdraw at any time. All information collected in the survey is strictly confidential. All published data will be anonymized and no individual persons or animals will be identifiable. Questionnaires will be identified by a unique code, which will match questionnaires and samples.

Please place the completed questionnaire in the pre-paid addressed envelope provided for return to the research team. Thank you.

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6. Please describe the ground within the enclosure for the animals *[please tick all that apply]*:

Solid floor with bedding ☐ Grass coverage ☐

Soil coverage ☐ Other *(please specify)*

7. Are the animals in a single species or mixed species enclosure?

Single species ☐ Mixed species ☐

If mixed species, please specify the species sharing the enclosure

8. Are the animals dewormed regularly as part of a health program?

YES / NO / Don't know

9. Please complete the table below about **any** deworming undertaken for this species:

	Adults	Whelping bitches	Pups
Name of dewormer used (trade name and active ingredients if known):			
Dose given (e.g. mg/kg, ml/kg or tablets/animal)			
Frequency of deworming (e.g. times per week/month/year)			
Date of last deworming (DD/MM/YY)			
Person responsible for deworming (e.g. keeper, vet, other)			

2. Is the zoo licensed as a Collection Centre for Animal By-Products?

YES / NO / Don't know

3. Within the last 12 months, please indicate which of the following diets have been fed to the animals *(tick all that apply):*

	Sheep	Lamb	Beef	Calf	Pig	Horse	Donkey	Goat	Poultry
Raw flesh from fallen stock (whole or part carcass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw liver or lungs (lights) from fallen stock	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw flesh from abattoir/butcher	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw liver or lungs (lights) from abattoir/butcher	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cooked flesh from butcher or cooked on-site	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cooked liver or lungs (lights) from butcher or cooked on site	☐	☐	☐	☐	☐	☐	☐	☐	☐

Commercially prepared food (dry/canned) ☐ Catering waste ☐

Fish ☐ Other (please specify) ☐

4. If used, please indicate how you store RAW meat intended for the animals *(tick all that apply)*:

Freezer ☐

Refrigerator ☐ Other *(please specify)*

Room temperature ☐ ☐

5. How does the zoo dispose of Specified Risk Material (SRM) and raw meat waste?

Incinerator on-site ☐ Other *(please specify)*

Rendering plant ☐ ☐

Incinerator off-site ☐

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10. Is testing of faecal parasites part of a routine health monitoring program for this species?

YES / NO / Don't know

11. If Yes, how often is this testing carried out?

12. Where is this test conducted?

On-site laboratory ☐ External specialist laboratory ☐

Local veterinary practice ☐ Other ☐

13. Are voided faeces from this species routinely collected and disposed? YES / NO / Don't know

14. If Yes, how are these faeces disposed?

15. Would you be willing to kindly participate in a second part of this study by collecting faeces samples from the animals using the kit provided? The samples are returned **FREE OF CHARGE** by post for testing for *Taenia* and *Echinococcus* tapeworms.

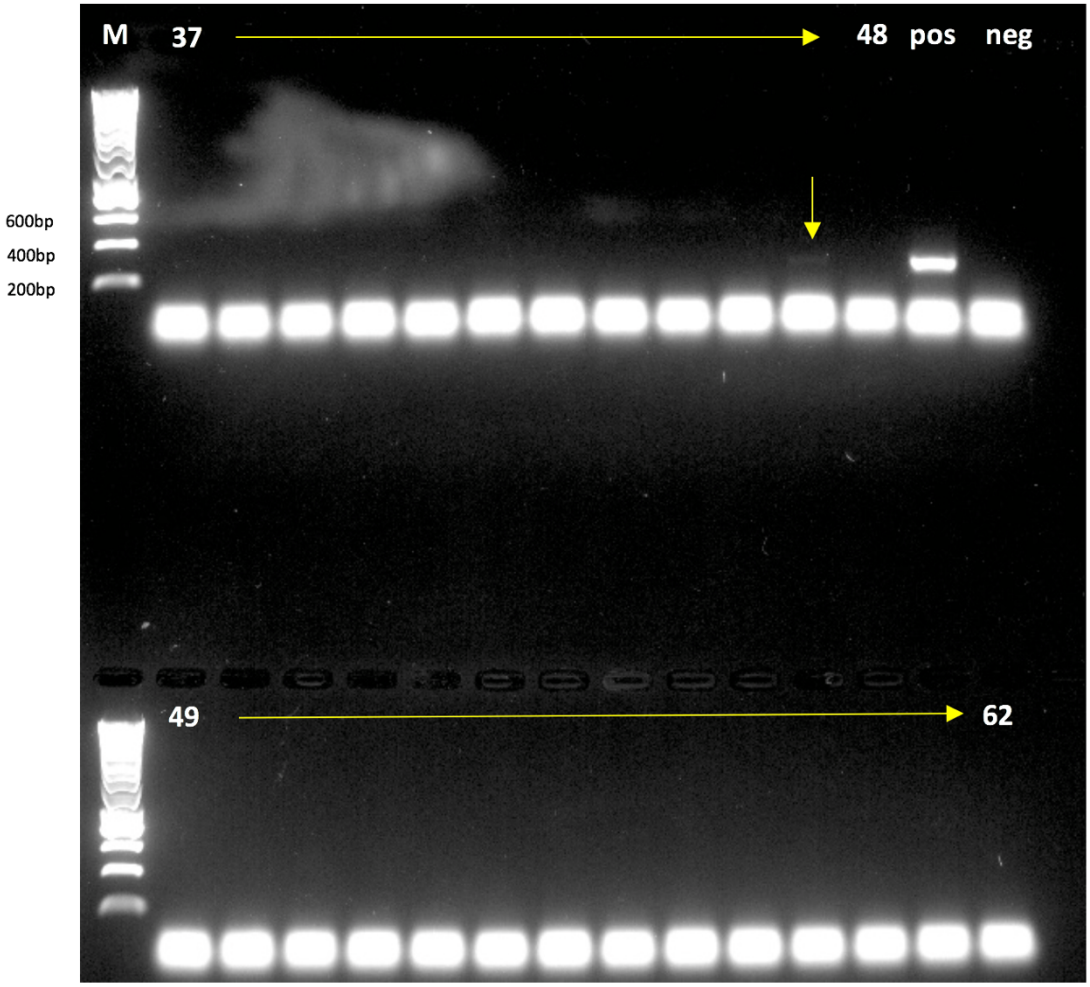
YES ☐ NO ☐

16. Would you be willing to kindly allow the lead investigator to visit the zoo to collect a further set of faeces samples from the animals at a pre-arranged date and time should this study be extended?

YES ☐ NO ☐

End of survey. Thank you for your valuable contribution. Your help is greatly appreciated.

Appendix II-d. Representative 1.5% agarose gel showing products of the E. granulosus G4 protocol developed by Lett (2013), which amplifies a species-specific 299bp segment within the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene, visible at sample 47/62. Marker ladder (M), positive E. equinus control (pos) and negative control (neg) are shown. The image brightness has been adjusted to allow visualisation of the feint sample band (yellow vertical arrow). This has also increased a PeqGREEN artifactual staining effect in the top left of the image.



Appendix II-e. Associations between coproELISA test outcomes and questionnaire variables in a cross-sectional survey of echinococcosis in UK zoo canids and hyaenids. Values represent outcomes of a two-sided Fisher's exact test with significance set at p<0.05.

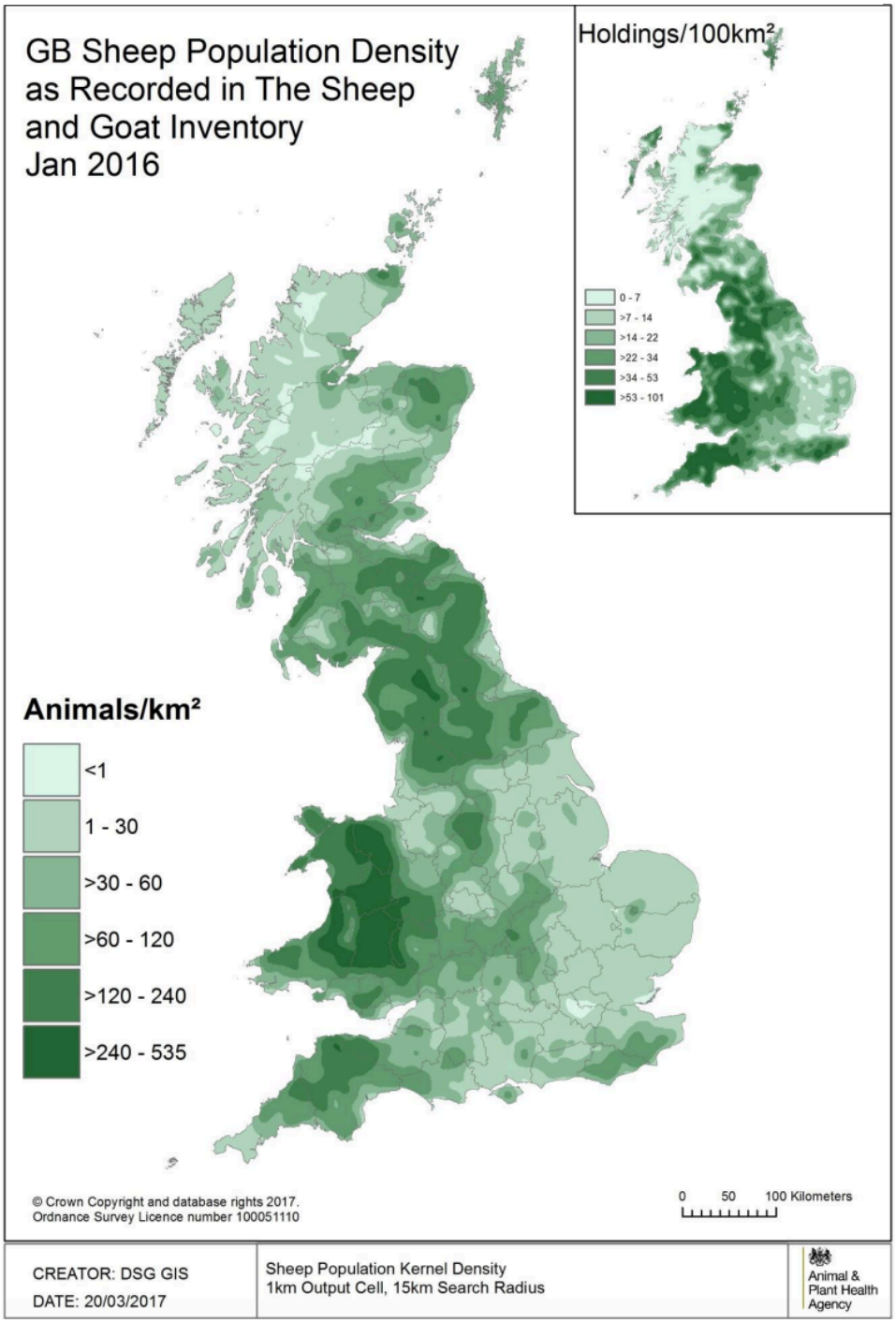
Variable		coproELISA positive
Species		
	Overall	0.216
	African Hunting Dog	0.418
	Bat-eared Fox	1.000
	Black-backed Jackal	0.122
	Bush Dog	1.000
	Corsac Fox	1.000
	Dhole	1.000
	Eastern Aardwolf	1.000
	European Grey Wolf	1.000
	Fennec Fox	1.000
	Iberian Wolf	0.330
	Maned Wolf	1.000
	Red Fox	1.000
	Spotted Hyena	1.000
Fallen stock (Meat)		
	Cattle/Sheep	0.554
	Other mammals	0.557
	Poultry	0.557
Fallen Stock (Viscera)		
	Cattle/Sheep	0.570
	Other mammals	0.108
	Poultry	1.000
Abattoir/butcher (Meat)		
	Cattle/Sheep	0.206
	Other mammals	0.118
	Poultry	0.604
Abattoir/butcher (Viscera)		
	Cattle/Sheep	0.298
	Other mammals	0.108
	Poultry	0.207
Cooked (Meat/Viscera)		
	Cattle/Sheep	0.298
	Other mammals	0.380
	Poultry	0.456
	Commercial/Specialist	0.296
Other		
	Catering waste	1.000
	Fish	1.000
	Rabbits/Rodents	1.000
	Fruit/Veg/Insects	0.554

Variable		coproELISA positive
Echinococcus risk		
	VH risk diet	1.000
	Any risk diet	1.000
	Low risk only	1.000
Raw food storage		
	Freezer	1.000
	Refrigerator	1.000
	Room temperature	1.000
Fallen stock disposal		
	Incinerator on-site	NA
	Incinerator off-site	0.456
	Rendering plant	1.000
	Disposal company	0.380
Enclosure flooring		
	Solid flooring	1.000
	Soil	0.557
	Grass	1.000
	Additional substrate	0.282
Faeces disposal		
	Pick up and dispose	NA
	Muck heap	1.000
	Waste disposal company	0.099
	Refuse bin	1.000
	Farmer's field	1.000
	Incinerator	0.555
Worming		
	Prophylactic worming	0.488
	Contain praziquantel	1.000
	Every 12 months	1.000
	Every 6 months	0.279
	Every 3 months	0.459
	Only if lab test positive	0.499
Laboratory testing		
	Every 12 months	1.000
	Every 6 months	0.511
	Every 3 months	1.000
Laboratory site		
	On-site	1.000
	External specialist	1.000
	Veterinary clinic	1.000

III. Appendix III: Chapter 5 Material

Appendix II-a. Map of UK NSA member regions including constituent counties in each region. Source: NSA, 2019.

Appendix III-b. Sheep population density in Great Britain (inset: number of sheep holdings in GB) correct to January 2016, collected for the sheep population report by the Livestock Demographic Data Group, APHA. Source: APHA, 2017.



Appendix III-c. HyData farm dog study participant information sheep issued with questionnaire and sampling kits.

HyData Farm Dog Study: Participant Information Sheet

Thank you for agreeing to take part in this study about the **husbandry and health care of farm dogs**, both working and non-working. The study is led by veterinary researchers at the University of Liverpool in collaboration with the National Sheep Association (NSA) as part of a larger 3-year research project into tapeworm infections in dogs and livestock in the UK. This information sheet gives details on the purpose, process and outcomes of this research. **Please read this information sheet carefully and feel free to raise any questions, comments or request clarification about any part of the study.**

What is the purpose of this study?

***Hy*Data** is a study to investigate the UK distribution of the dog tapeworm, *Echinococcus granulosus.* This small parasite causes hydatid disease, resulting in considerable economic loss to the UK livestock industry and posing an infection risk to many species, including humans. This parasite is present in the UK, but information about where in the country it is found is currently lacking.

The tapeworm does not often pose a disease risk to dogs, but infection spread by them to livestock, such as sheep and cattle can lead to significant production losses for livestock farmers. In the rare event that a person is infected, this can result in debilitating disease that is difficult to treat.

Through our study, we hope to find out more about *Echinococcus* in dog populations and livestock. Alongside farm dogs, the overarching HyData project also includes sampling *Echinococcus* in sheep and cattle at slaughter, hunting hounds and canine zoo animals in the UK. With this information, we aim to improve and inform on the welfare, safety and health of people, at-risk dog groups and livestock in our country.

Who is conducting the study and who is it funded by?

The study is led by researchers at the University of Liverpool and is funded by the Biotechnology and Biological Sciences Research Council (BBSRC) and the University. The study is conducted with full ethical approval from the University of Liverpool Veterinary Research Ethics Committee.

Why have I been chosen to take part?

All UK NSA members that own dogs on-farm, both working and non-working are invited to take part in the study. Collaboration with NSA members offers an ideal opportunity to work with a large membership base of sheep farmers interested in best practice in animal health.

What does taking part involve?

You are being invited to help in two ways; firstly, to complete a short questionnaire, which should take no more than 10 minutes. Secondly, you are asked to submit a sample of faeces from the dogs you have on the farm, via a sampling kit we provide free-of-charge. No direct contact with the dogs is involved in the process.

1

Do I have to take part?

Taking part is completely voluntary and you are free to withdraw from the study at any time.

Will taking part in the study be confidential?

Yes. Any information you give, including contact details to receive your sampling kit, will be held in strict confidence in a secure, password-protected database at the University of Liverpool in accordance with GDPR regulations and the Data Protection Act (2018). All information will be **fully anonymised** and will be destroyed within 5 years of study completion. Questionnaires will be identified by a unique code, which will be used to link samples to the right questionnaire. Your personal details, your dog(s) and farm address will not be identified through published research. **By providing contact details, completing the questionnaire and submitting samples, you are giving informed consent for your data to be included in the study.**

What will happen to the information and samples I provide?

The questionnaire data will be safely stored and later analysed to investigate the national distribution of *Echinococcus*, and possible risk factors for infection in a rural setting. Faeces samples from the dog(s) will be tested in the laboratory to see if it or they contain the *Echinococcus* parasite. Although our research doesn't represent a validated diagnostics service, like that of a commercial laboratory, we think the information we are gathering is important and we will report the results of the laboratory tests to you free of charge via the contact details you provide. We encourage you to discuss the findings of the test with your veterinary surgeon and take the project information sheet with you when you do.

What are the benefits and risks of taking part in the study?

Collection of samples is not expected to cause any undue health risks above those encountered during normal daily care of your farm dogs and disposal of their faeces. We do recommend that you use the disposable gloves provided during sample collection and follow the instructions as show on the kit. Taking part will help us gather vital information that may help protect your dogs, the livestock and people that share our countryside from a potentially harmful parasite.

What will be the outcome of the study?

We aim to publish the results of the study in appropriate scientific journals, present the findings at scientific meetings, and work with parasitology experts to develop evidence-based advice on tapeworm protection for dog owners. Again, no participants will be identifiable from any published or presented results.

What if I no longer wish to take part?

If you change your mind and no longer wish to take part, even after you have answered the questionnaire and submitted samples, we will delete your answers and destroy the samples so that they are not included in the study. Simply contact us to request this.

What should I do if I have a problem?

If you are unhappy with any aspect of the study or encounter a problem, please inform the lead researcher, Marisol Collins (details below) or the Principal Investigator, Dr. Eleni Michalopoulou on 0151 795 6057 and we will endeavour to help. If you have a complaint that you do not wish to share

with the project team, please contact the Research Governance Officer of the University of Liverpool at ethics@liv.ac.uk with the name and description of the study, so it can be identified.

Yes! I want to help the study by taking part, what next?

If you would like to take part in the study, please contact the lead veterinary researcher via email or telephone via the details below for advice on receiving your questionnaire and sampling kit:

CONTACT DETAILS

Ms. Marisol Collins MRCVS
Institute of Infection and Global Health
University of Liverpool
Leahurst Campus
CH64 7TE
Tel: 0151 795 6040
hydata@liv.ac.uk

Thank you for your valuable contribution

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Appendix III-d. HyData farm dog study questionnaire for a cross-sectional survey of NSA member farms on echinococcosis in farm dogs.

*Hy*Data Farm Dog Survey 2018

Thank you for agreeing to take part in this study. Your participation is greatly valued and will help us to better understand the distribution of the *Echinococcus* tapeworm in dogs and livestock in the UK.

As part of our research, we are inviting randomly selected NSA members with farm dogs to complete a questionnaire about the husbandry and healthcare of their dog(s) and submit a sample of fresh faeces from the dog(s) for *Echinococcus* testing in our laboratory.

Before completing the survey, please read the accompanying Participant Information Sheet carefully, complete the Participant Consent Form attached to this questionnaire and read the instructions below. We ask that a person who is responsible for the daily care of the dogs completes the questionnaire and consent form.

How to complete the survey

The survey is divided into two sections and should take approximately 10 minutes to complete:

Section 1: About your dog(s) Here, questions relate to the number of dogs, their breeds, sex and age.

Section 2: Husbandry at the farm This section includes questions about the diet of the dog(s), their feeding regime, exercise facilities, deworming procedures and faeces disposal.

Please answer questions in BLOCK CAPITALS in the text box provided **OR** by circling the appropriate answer if asked YES / NO / Don't know **OR** by placing a tick in the box ☑ where appropriate. If you change your answer or make a mistake, please fill in the first box completely ■ and put a clear tick in the correct box.

Taking part in this study is entirely voluntary and you are able to withdraw at any time. All information collected in the survey is strictly confidential. All data will be anonymised and no individual persons or dogs will be identifiable from any published results. Questionnaires will be identified by a unique code, which will match the questionnaires and samples and will be used to ensure that further invitations are not sent to existing participants.

Please place the completed questionnaire and samples in the pre-paid addressed envelope provided for return to the research team. If you have any queries, please contact the lead researcher Marisol Collins via email at hydata@liv.ac.uk or phone on 0151 795 6040. Thank you.

1

Date DD / MM / 2018

Section 1: About your dog(s)

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1. How many dogs do you keep on the farm?

2. Please complete the table below (entering number of dogs in each individual category):

	Total	Males (6 months old or over)	Females (6 months old or over)	Pups (under 6 months old)	Unknown age
Working					
Non-working					

Section 2: Husbandry, diet and worming of your dog(s)

1. Please describe the environment given for the dogs to exercise *[please tick all that apply]*:

Fenced grass yard ☐ Fenced field/area shared with livestock ☐

Fenced concrete yard ☐ Tethered in unfenced area ☐

Open land with public access ☐ Other (please specify)

2. Have you placed any public notices on public access land relating to keeping pet dogs wormed and/or on the lead?

YES ☐ NO ☐

3. Have you ever known you dog(s) to scavenge remains of fallen stock on farm land prior to its collection?

YES, frequently (e.g. daily, weekly or monthly) ☐ NO ☐

YES, rarely (e.g. yearly or less) ☐ Unknown ☐

We would now like to know about the type of diets you feed your dog(s). This includes any raw meat diet (and animal species fed), commercial and non-commercial foods and how they are stored at home.

4. How many meals are fed to the dog(s) over 24 hours?

5. Within the last 12 months, please indicate any of the following diets that have been fed to your dog(s) *(tick all that apply):*

Commercial dog food (dry/canned) ☐ Catering/food waste/kitchen scraps ☐

Fish ☐

	Sheep	Lamb	Beef	Calf	Pig	Horse	Donkey	Goat	Poultry
Raw flesh from fallen stock (any source, whole or part carcass)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw liver or lungs (lights) from fallen stock (any source)	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw flesh from abattoir/butcher	☐	☐	☐	☐	☐	☐	☐	☐	☐
Raw liver or lungs (lights) from abattoir/butcher	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cooked flesh from butcher or cooked on-farm	☐	☐	☐	☐	☐	☐	☐	☐	☐
Cooked liver or lungs (lights) from butcher or cooked on-farm	☐	☐	☐	☐	☐	☐	☐	☐	☐

Other (please specify) ☐

6. **If used**, please indicate how you store RAW meat intended for feeding your dog(s) *(tick all that apply)*:

Freezer ☐ Room temperature ☐

Refrigerator ☐ Other *(please specify)* ☐

7. What approved method(s) do you have for disposal and collection of fallen stock?

Abattoir ☐ Hunt kennel ☐ Knacker ☐

Rendering plant ☐ Maggot farm ☐ Zoo ☐

Incinerator ☐ Other approved site ☐

8. Do you use the National Fallen Stock Company NFSCo? YES ☐ NO ☐

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9. Do you deworm your dog(s) on a regular basis? YES / NO / Don't know

10. Please indicate which information source you most use to make choices about the brand of dewormer, dose and frequency you use *(please tick one only):*

Veterinary surgeon ☐ Pet shop ☐ Online source ☐

NSA advice ☐ Other ☐

11. Please complete the table below about **any** deworming undertaken for your dog(s):

	Working Dogs (6 months or older)	Non-working dogs (6 months or older)	Pups (less than 6 months)	Pregnant or whelping bitches
Name of dewormer used				
Dose given (e.g. mg/kg, ml/kg or tablets/dog)				
Frequency of deworming (e.g. times per week/month/year)				
Date of last deworming (DD/MM/YY)				
Person responsible for deworming (e.g. owner, farm employee, vet)				

12. Do you or others responsible for the care of the dog(s) routinely pick up the dog(s) faeces for disposal? YES / NO / Don't know

13. If you pick up the dog(s) faeces, how often and how are they then disposed?

Please describe collection routine, method and disposal site

14. Would you be willing to kindly participate in a **second part** of this study by collecting faeces samples from the dogs using the kit provided? The samples are returned **FREE OF CHARGE** by post for **anonymized** testing for *Echinococcus* tapeworm.

YES ☐ NO ☐

15. Would you be willing to kindly allow the lead investigator to visit your farm to collect a further set of faeces samples from the dog(s) at a pre-arranged date and time for **anonymized** testing for *Echinococcus* tapeworm?

YES ☐ NO ☐

16. Would you be willing to be contacted by the NSA to participate in future research projects?

YES ☐ NO ☐

End of survey. Thank you for your valuable contribution. Your help is greatly appreciated.

Appendix III-e. Representative 1.5% agarose gel showing products of the E. granulosus G1 protocol developed by Boufana et al., (2013) amplifying a species-specific 226bp segment within the NADH dehydrogenase subunit 1 (ND1) mitochondrial gene, visible at sample 16/22. Marker ladder (M), positive E. granulosus control (pos) and negative control (neg) are shown. The image shows two halves of the same gel, split for imaging purposes, with brightness adjusted in the right image to allow visualisation of the feint sample band.

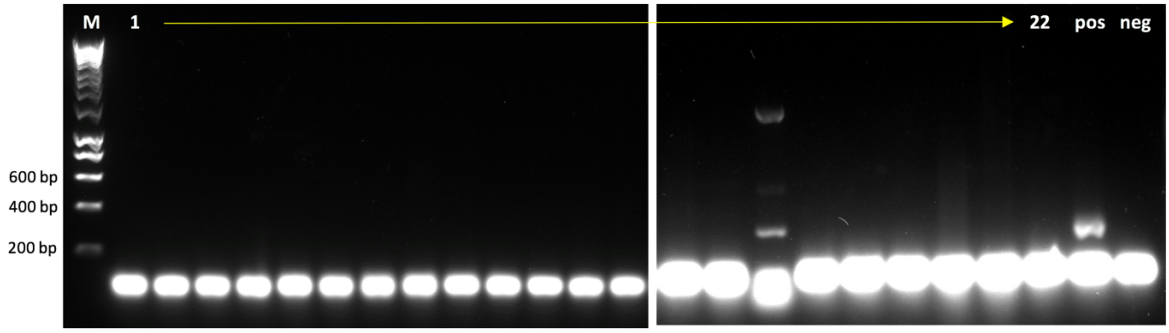


Table III-f. Associations between coprodiagnostic test outcomes and questionnaire variables in a cross-sectional survey of echinococcosis in farm dogs on NSA member farms. A grey box denotes as significant value (p<0.05).

		two-sided Fisher's exact (p)		
Diet type		**coproELISA**	**coproPCR G1**	**Any copro-test positive**
Fallen stock (Meat)				
	Cattle/Sheep	0.176	0.327	0.679
	Other mammals	0.45	0.246	0.417
	Poultry	NA	NA	NA
Fallen Stock (Viscera)				
	Cattle/Sheep	0.074	1	0.229
	Other mammals	0.174	0.13	0.234
	Poultry	NA	NA	NA
Abattoir/butcher (Meat)				
	Cattle/Sheep	0.569	1	0.322
	Other mammals	1	1	1
	Poultry	NA	NA	NA
Abattoir/butcher (Viscera)				
	Cattle/Sheep	1	1	1
	Other mammals	1	1	1
	Poultry	NA	NA	NA
Cooked (Meat)				
	Cattle/Sheep	0.324	1	0.663
	Other mammals	1	1	1
	Poultry	1	1	1
Cooked (Viscera)				
	Cattle/Sheep	1	0.44	1
	Other mammals	NA	NA	NA
	Poultry	NA	NA	NA
Other	Commercial	NA	NA	NA
	Catering waste	1	1	0.74
	Fish	1	0.52	1
	Other			
***E. granulosus* risk**				
	Very high risk diet	0.178	1	0.467
	Any risk diet	0.232	0.38	0.737
	Low risk diet only	0.232	0.38	0.737
Raw food storage				
	Freezer	1	1	0.33
	Refrigerator	0.321	0.246	0.45
	Room temperature	1	1	1
Fallen stock disposal				
	Incinerator	1	1	1
	Rendering plant	1	1	0.659
	Knackers	1	0.39	1
	Abattoir	NA	NA	NA
	Hunt kennel	1	0.57	0.659
	Maggot farm	NA	NA	NA
	Zoo	1	1	1
	Other	1	1	1
	NFSCo member	1	0.65	0.314

Table III-e continued.

		two-sided Fisher's exact (p)		
Diet type		**coproELISA**	**coproPCR G1**	**Any copro-test positive**
Sites of access				
	Fenced grass yard	0.243	0.647	0.07
	Fenced concrete yard	0.699	0.659	1
	Fenced field/area with livestock	0.658	0.619	0.244
	Open land with public access	1	1	1
	Tethered	1	1	1
	Roads	NA	NA	NA
	Potential livestock/public risk	0.664	0.598	0.414
Scavenging				
	Frequent	0.444	1	1
	Occasional	1	1	1
	None	0.699	0.655	1
	Any	0.713	0.684	1
	Don't know	1	1	1
Faeces disposal				
	Pick up and dispose	0.439	0.198	0.505
	Rendering plant	NA	NA	NA
	Muck heap	0.129	0.056	0.183
	with SRM	NA	NA	NA
	Offal skip	NA	NA	NA
	Burning on site	NA	NA	NA
	Farmer	NA	NA	NA
	Waste disposal company	1	1	1
	Slurry pit	1	1	1
	Refuse bin	0.325	0.57	0.175
	Buried	1	0.349	0.57
	Don't know	1	**0.014**	0.051
	Do not collect	0.246	**0.034**	0.737
NSA region				
	All	0.347	0.265	0.109
	Scotland	1	0.221	1
	Northern	0.089	1	0.33
	Wales	0.548	0.077	**0.035**
	Central	1	1	1
	Eastern	0.444	1	0.56
	South West	0.571	0.579	0.312
	South East	0.321	1	0.417
	Marches	1	0.327	0.413
	Northern Ireland	0.569	0.52	1
Worming	Contain praziquantel	1	1	1
	According to manufacturer	0.433	**0.035**	0.079

IV. Appendix IV: Chapter 6 material

Appendix IV-a Hydatid study sampling instructions

Participant Information Sheet

***Hy*Data 2017: *Echinococcus* tapeworm infection (hydatid disease) in sheep and cattle at slaughter in England and Wales**

You are invited to take part in a sampling survey about hydatid disease, caused by the tapeworm parasite *Echinococcus granulosus*, in sheep and cattle identified during post-mortem meat hygiene inspection. This forms part of a 3-year research project into tapeworm infections in dogs and livestock in the UK. It is important that you understand fully the purpose, process and outcomes of this research before deciding if you wish to take part. **Please read this information sheet carefully and feel free to raise any questions, comments or request clarification about any part of the study.**

What is the purpose of this study?

This study is part of a research project on ***Echinococcus***, a tapeworm parasite of dogs, which can also infect livestock, horses and people. This parasite is present in the UK, but information about where in the country it is found, and the risk this poses to people and livestock is currently lacking.

E. granulosus presents a significant risk to human health and is an important source of economic loss for livestock industries, associated with poor growth, reduced meat and milk production and rejection of organs at meat inspection. Food Standards Agency (FSA) records report hydatidosis in an estimated 0.1-0.2% of sheep and cattle carcasses examined post-mortem in England and Wales, however, this is currently not supported or validated by laboratory testing. Currently, there is little information about the distribution of the parasite in both definitive canine hosts and intermediate livestock hosts in the UK. There is concern among medical and veterinary parasitologists that hydatidosis may be a re-emerging disease in the UK, and the true extent of the human and animal burden of this disease is underestimated. In the rare event that a person is infected, this can result in debilitating disease that is difficult to treat.

The study has two broad aims. Firstly, to investigate the national distribution of the parasite by collecting tissue samples to identify *Echinococcus* by molecular and genetic testing in sheep and cattle raised throughout England and Wales. With this information, we aim to improve and inform on the safety and health of people, domestic animals and livestock in our country. The second aim is to use the laboratory data to validate the statutory visual inspection process for hydatidosis undertaken at slaughter by trained Meat Hygiene Inspectors (MHIs) and Official Veterinarians (OVs) in England and Wales. This is a collaboration with the FSA, who are currently undertaking a wider review of current meat hygiene inspection processes in abattoirs. As a pilot validation exercise, the study will give valuable insight into the feasibility of applying confirmatory diagnostics to other diseases identified at post-mortem, improving the robustness and reliability of the meat inspection process.

Who is conducting the study and who is it funded by?

1

The study is a collaboration between the Food Standards Agency (FSA) and researchers at the Institute of Infection and Global Health at The University of Liverpool, and is funded by the Biotechnology and Biological Sciences Research Council (BBSRC). The study is conducted with full ethical approval from The University of Liverpool Committee on Research Ethics.

Why have I been chosen to take part?

Twenty-five large abattoirs from England and Wales will be included in the study, which together account for 80% of the slaughter throughput for sheep and cattle in these devolved administrations (based on FSA 2015-2016 data). You have been chosen to take part as you undertake meat hygiene inspection processes at one of the selected abattoirs. All Meat Hygiene Inspectors at participating abattoirs will be invited to take part in the study.

What does taking part involve?

You are invited to help in two ways during the study; Firstly, to collect a small sample of any liver or lung tissue you identify as containing hydatid cysts at post-mortem inspection and intend to remove as animal by-product (ABP). When hydatid tissue is identified and collected from a carcass, we also ask that you collect a sample of the next 3 tissues you identify as containing ANY disease (we do not need to know the identity of disease material), for example, *Cysticercus tenuicollis* cysts in sheep, abscesses, liver fluke lesions etc. We ask you to collect these extra samples so we can validate the hydatid identification process in the laboratory. Secondly, we ask you to provide any carcass identification details for the hydatid samples collected i.e. individual animal tag number and herd mark in cattle, and any information on identifiers for sheep. Instructions on all sample and data collection are provided with the sampling kit. The study will run for a period of 6 months, or less if the total required number of samples for the study is collected sooner.

Collection kits and clear instructions are provided for all sample collections, and the kits will be returned to the research laboratory by pre-paid First Class Royal Mail post. A stock of new kits will be provided to each abattoir at intervals to ensure you have sufficient for sample collection.

Do I have to take part?

You will be invited to participate by the FSA and the University of Liverpool research team. Taking part is completely voluntary and you are free to withdraw from the study at any time.

Will taking part in the study be confidential?

Yes. Any information you give will be held in strict confidence in a secure, password-protected database at the University of Liverpool in compliance with the Data Protection Act (1998). All information will be fully anonymised and will be destroyed within 5 years of study completion. Any information collected will be identified by a unique code. Your personal details are not requested, nor will any individuals be identifiable from the data we collect. The name and exact location of participating abattoirs will not be identifiable through published research. **By completing this questionnaire and providing samples, you are giving informed consent for your data to be included in the study.**

What will happen to the information and samples I provide?

The abattoir data will be safely stored and later analysed to investigate the national distribution of *Echinococcus*, and possible risk factors for infection, alongside other data collected. Raw data will be kept for a maximum of 5 years

from collection. Tissues samples will be tested in the laboratory to see if they contain the *Echinococcus* parasite, confirming the visible lesions as hydatid or not. The DNA extracted from the tissues to identify the parasite will be kept frozen for a maximum of 5 years, after which it will be destroyed.

What are the benefits and risks of taking part in the study?

By taking part in the study, you will help us to gather vital information to help improve the robustness and reliability of the meat hygiene inspection process, and explore the distribution of this important disease in the UK. Taking part does not involve taking any additional risks during the routine meat hygiene inspection and day-to-day work at the abattoir.

What will be the outcome of the study?

The study results will help the FSA to validate the inspection process for this disease, and will give valuable insight into the feasibility of applying confirmatory diagnostics to other diseases identified at post mortem. The data on national distribution of hydatid disease in livestock will form part of the lead researcher's PhD thesis and will be published in appropriate scientific journals and presented at scientific meetings. No participants will be identifiable from any published or presented results.

What if I no longer wish to take part?

If you change your mind and no longer wish to take part, even after you have submitted samples, we will destroy the samples so that they are not included in the study. Simply contact us to request this.

What should I do if I have a problem?

If you are unhappy with any aspect of the study or encounter a problem, please inform us by contacting the Principal Investigator, Dr. Phil Jones on 0151 795 6056 and we will endeavour to help. If you have a complaint that you do not wish to share with the project team, please contact the Research Governance Officer of The University of Liverpool at ethics@liv.ac.uk with the name and description of the study, so it can be identified.

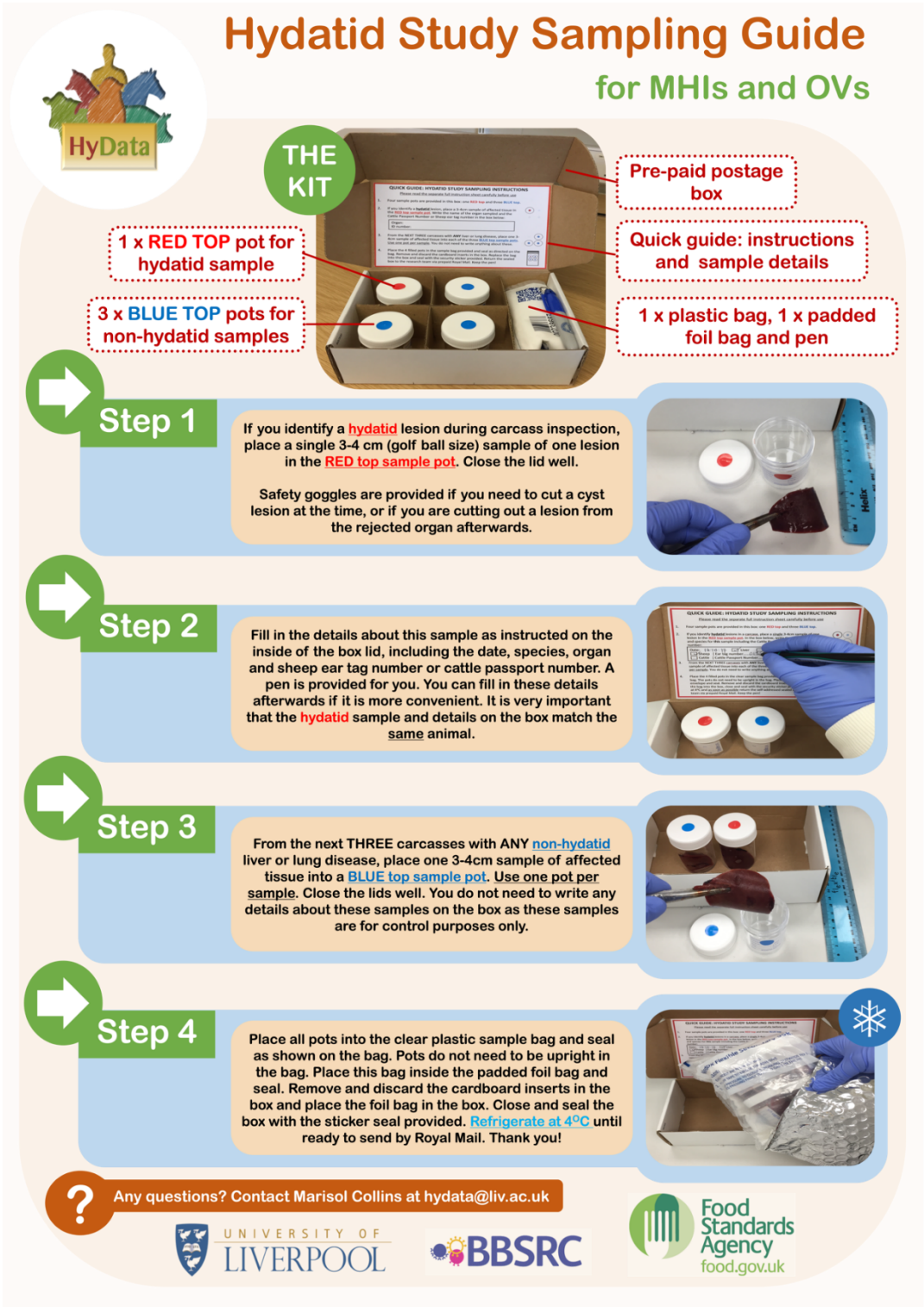
Who can I contact if I have further questions?

If you have any questions or comments relating to the study, please contact the veterinary investigator:

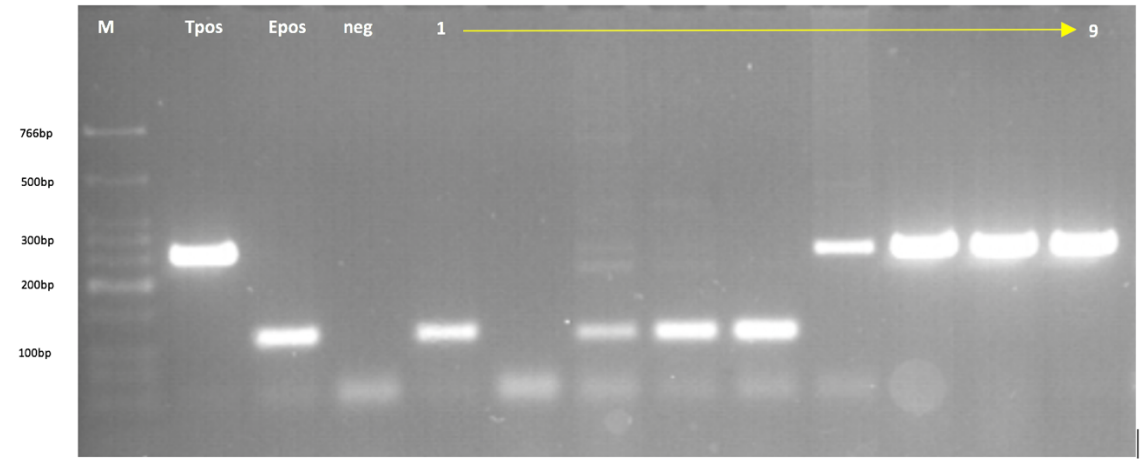
Dr. Marisol Collins
Institute of Infection and Global Health
School of Veterinary Science
Leahurst
Cheshire
CH64 7TE
Tel: 0151 795 6040
collinsm@liv.ac.uk

Thank you for your valuable contribution

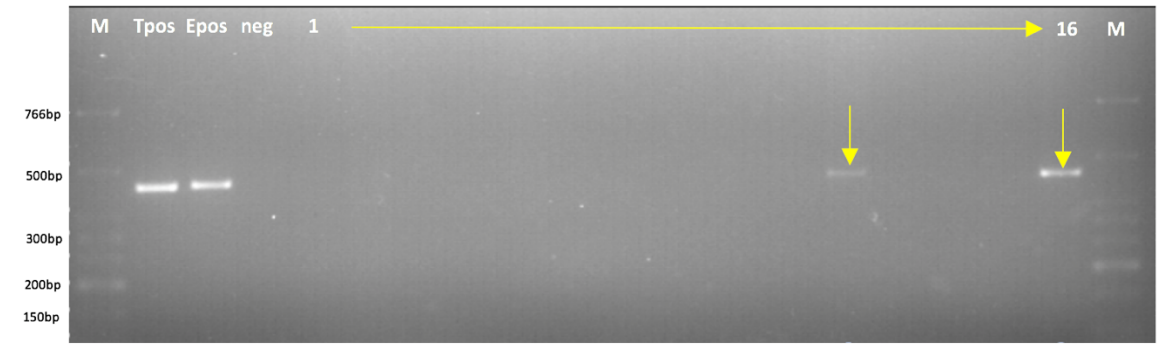
Appendix IV-b. Abattoir sampling advice



Appendix IV-ci. Representative 1.5% agarose gel showing products of the multiplex PCR protocol developed by Trachsel et al. (2007) amplifying an Echinococcus spp.- specific 117bp fragment of the small subunit of ribosomal RNS (rrnS) visible at samples 1,3,4,5/9 and a Taenia spp. -specific 267bp fragment of rrnS visible at samples 6-9/9. Marker ladder (M), positive Taenia DNA (Tpos) and Echinococcus DNA (Epos) controls and negative control (neg) are shown.



Appendix IV-cii. Representative 1.5% agarose gel showing the product of the PCR protocol developed by Bowles et al. (2007) amplifying an ~450bp fragment of the mitochondrial cox1 gene, visible at samples 12,16/16. Sequencing confirmed these to be E. graulosus. Marker ladder (M), positive Taenia DNA (Tpos) and Echinococcus DNA (Epos) controls and negative control (neg) are shown.



Appendix IV-d. a) An intact unilocular hydatid cyst from a bovine lung; b) stained section with H&E (x4 magnification) with the visible acellular laminar membrane c) section from the same lesion (x4), stained with PAS, demonstrating differential staining of the laminar membrane, a diagnostic feature of Echinococcus spp. Lesions; d) T. hydatigena cyst from an ovine liver; d) stained section of (c with H&E) (x10 magnification) showing a cross-section of the developing Cysticercus tenuicollis.